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Metabolism of omega-3 fatty acids in carriers of apolipoprotein E epsilon 4

By
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Thesis presented at the Faculty of medicine and health sciences
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Résumé

Métabolisme des acides gras oméga-3 chez les porteurs de l'apolipoprotéine E epsilon 4

Par Janani Balakrishnan, Programmes de physiologie

Mémoire présentée à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de maitre ès sciences (M.Sc.) en physiologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Selon l'organisation mondiale de la santé, 50 millions de personnes vivent avec la démence, où la maladie d'Alzheimer représente 60 à 70% de ces cas. Être porteur de l'allèle epsilon 4 de l'apolipoprotéine E (APOE4) est le principal facteur de risque génétique de la maladie d'Alzheimer tardive. Des études ont montré qu'une alimentation riche en acides gras (AG) oméga-3 comme l'acide docosahexaénoïque (DHA) et l'acide eicosapentaénoïque (EPA) peut réduire le risque de développer la maladie d'Alzheimer tardive. Cependant, l'avantage de la consommation d'AG oméga-3 n'est pas observé chez les porteurs de l'APOE4. De plus, des études ont démontré que le cerveau peut capter les AG lorsqu'ils sont estérifiés sous forme de lysophosphatidylcholine (LPC) ou lorsqu'ils ne sont pas estérifiés (FFA). Ainsi, notre hypothèse de recherche est qu'après avoir consommé des suppléments d'AG oméga-3 pendant six mois, l'augmentation de la concentration d'AG oméga-3 plasmatiques des porteurs de l'APOE4 n'augmente pas dans les LPC et FFA plasmatique par rapport aux non-porteurs de l'APOE4. Pour étudier cette hypothèse, nous avons analysé les concentrations plasmatiques de DHA et d'EPA dans les LPC, les phosphatidylcholines (PC), les phosphatidyléthanolamines (PE), les FFA, les triglycérides (TG) et les esters de cholestérol (CE). Les lipides totaux dans le plasma ont été extraits, puis séparés en CE, TG, FFA et phospholipides totaux en utilisant une chromatographie sur couche mince (CCM). Les PL ont par la suite été séparés en PE, PC et LPC en utilisant une deuxième plaque de CCM. Après avoir été convertis en ester méthylique d'AG, les AG méthylés ont été analysés par chromatographie en phase gazeuse. Après six mois de supplémentation en AG oméga-3, les concentrations plasmatiques de DHA et d'EPA ont augmenté dans tous les compartiments plasmatiques analysés. De plus, il y avait une tendance à ce que le DHA dans les CE soit plus élevé chez les porteurs de l'APOE4 comparativement aux non-porteurs. L'indice de masse corporelle a également affecté la réponse à la supplémentation. En effet, le ΔDHA et ΔEPA (pré - post concentration) n'a pas augmenté autant dans la LPC des individus ayant un indice de masse corporelle (IMC) élevé (>25,2 kg/m^2) comparativement à ceux avec un IMC <25,2 kg/m^2. Par conséquent, les participants du groupe à IMC élevé avaient moins de DHA disponible dans le compartiment plasmatique que le cerveau peut capter. En conclusion, notre hypothèse de recherche est infirmée ce qui suggère qu'une supplémentation en AG omega-3 pourrait bénéficier les porteurs de l'APOE4 puisque le DHA et l'EPA augmentent dans les compartiments plasmatiques qui permet la capture des AG omega-3 par le cerveau.

Mots-clés : Acides gras oméga-3, métabolisme des acides gras, APOE4, déclin cognitive

Summary

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According to world health organization, 50 million individuals live with dementia, where Alzheimer's disease represents 60 to 70% of those cases. Carrying the apolipoprotein E epsilon 4 allele (APOE4) is the main genetic risk factor for late onset Alzheimer's disease. Studies have found that consuming a diet rich in omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can decrease the risk of developing Alzheimer's disease. However, the benefit of consuming omega-3 fatty acids is not seen in APOE4 carriers. Additionally, studies have demonstrated that brain can take-in fatty acids in the lysophosphatidylcholine (LPC) and free fatty acid (FFA) compartments. Therefore, we hypothesized that after consuming omega-3 fatty acid supplements for six months, APOE4 carriers' plasma omega-3 fatty acid levels would not increase in the LPC and FFA plasma lipid compartments compared to non-carriers of the APOE4. To investigate this, we analysed DHA and EPA concentrations in the following plasma lipid compartments: LPC, phosphatidylcholine (PC), phosphatidylethanolamine (PE), FFA, triglyceride (TG) and cholesteryl esters (CE). Firstly, lipids were extracted from the plasma and then separated into CE, TG, FFA, and PL using thin layer chromatography. PL were further separated into PE, PC and LPC using a second thin layer chromatography plate. The fatty acids in the separated lipid compartments were converted to fatty acid methyl ester to be analysed using gas chromatography. In the current study, post-omega-3 fatty acid supplementation for six months, plasma DHA and EPA levels increased in all the lipid compartments analyzed. Additionally, there was a genotype trend for DHA in CE, where APOE4 carriers had higher levels of DHA compared to non-carriers before and after omega-3 supplementation. When comparing ΔDHA and ΔEPA (pre – post concentration) in the different lipid compartments between low and high body mass index (BMI) groups (<25.2 kg/m2 and >25.2 kg/m2, respectively) and genotype, plasma concentration of DHA and EPA in LPC of individuals in the high BMI group did not increase as much as those in the low BMI group after taking omega-3 fatty acids. Therefore, individuals in the high BMI group had less DHA available in the lipid compartment the brain can take-in. In conclusion, our research hypothesis is rejected as our results suggests supplementation with omega-3 fatty acids could benefit APOE4 carriers since DHA and EPA levels are higher in the plasma compartments where uptake of omega-3 fatty acids are possible by the brain.

Key words: Omega-3 fatty acids, fatty acid metabolism, APOE4, cognitive decline, Alzheimer's disease

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List of abbreviations

ALA	Alpha-linolenic acid
APOE2	Epsilon 2 allele of apolipoprotein E
APOE3	Epsilon 3 allele of apolipoprotein E
APOE4	Epsilon 4 allele of apolipoprotein E
ARA	Arachidonic acid
BMI	Body mass index
CE	Cholesterylester
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FFA	Free fatty acid
HDL	High density lipoprotein
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LPC	Lyso-phosphatidylcholine
VLDL	Very low density lipoprotein
Mfsd2a	Major facilitator superfamily domain containing 2A
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid
PLA2	Phospholipase A2
PSEN1	Presenillin 1
PSEN2	Presenillin 2
TG	Triglyceride
TLC	Thin layer chromatography

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1. Introduction

1.1 Cognitive Impairment - Alzheimer's Disease

It is estimated 50 million people live with dementia worldwide. In 20 years, this number will nearly double ("Dementia statistics," 2020). Dementia is characterized by progressive decline of cognitive functions such as memory, language, apraxia, agnosia, and problem solving that can affect everyday tasks (Morris, 1996). Alzheimer's disease is the main cause of dementia, and accounts for more than 75% of the dementia cases (J. C. Morris, 1996). The salient characteristic of Alzheimer's disease is impairment of the memory. Personality changes is another symptom that indicates the presence of the disease. In the early stages of the disease, forgetfulness, slight temporal and geographical disorientation and miscalculating household finances are common. However, they will still be able to live independently as social and self-care abilities are normally intact. As the disease progresses, symptoms worsen over time where language comprehension, ability to handle money, ability to do everyday tasks like washing dishes and becomes difficult. Delusion, hyperactivity, physical aggression, and hallucinations can also be seen. In much more advanced stages, trouble speaking, walking, and swallowing which leads being bedbound (Morris, 1996). The neuropathology of Alzheimer's disease is characterized by neurofibrillary tangles, senile and neuritic plaques, and loss of synapses (Perl, 2010). Neurofibrillary tangles are high levels of tau protein phosphorylated abnormally. Senile or neuritic plaques are accumulations of amyloid--β protein at the core and is surrounded by abnormally formed neurites (Perl, 2010). Hallmarks of the neuropathology of Alzheimer's disease are the aggregation of neuritic amyloid plaques, and neurofibrillary tangles in the extracellular space (Cacace, et al., 2016; Jahn, 2013). These interfere with neurons ability to communicate with other neurons (synapses) and they eventually trigger neuroinflammation and degeneration of neurons (Cacace et al., 2016). These changes can begin in the entorhinal cortex and hippocampus and spread to other regions in the brain such as temporal, and parietal (Jahn, 2013). Delusions are associated with atrophy of the hippocampus. The most frequent behavioural symptom seen in Alzheimer's patients is

apathy which is characterized by the inability to have a motivated response (Boublay et al., 2016). During autopsy of Alzheimer's disease patients, it was found apathy is correlated with a build-up of plaque and neurofibrillary tangles in the anterior cingulate cortex, and rarely associated with parietal and temporal regions of the brain (Boublay et al., 2016). There are multiple risk factors to developing Alzheimer's disease, where some factors are unmodifiable like age and carrying the epsilon 4 allele of the apolipoprotein E (APOE4) and some are modifiable like a diet poor in omega-3 fatty acids. The next section will focus more the two main factors increasing the risk of developing Alzheimer's disease.

1.1.1 Age and Alzheimer's disease

The main risk factor for developing Alzheimer's disease is old age (Askarova et al., 2020). First symptoms of late onset Alzheimer's disease present after the age of 65 years. According to Statistics Canada, the population of Canada has increased greatly from 30.7 million to 37.1 million between the year 2000 and 2018. It is projected to increase to 55.2 million in the next fifty years. The ratio of individuals above the age 65 years was around 17.2% in 2018 compared to the population. This ratio has been gradually increasing and is expected to increase to 21.4%-23.4% of the population (Statistics Canada, 2019). Two things are seen in the aging population. There is a decline of physiological functions and an increase in the proportion of individuals with a disease (Abrass, 1990). In the aging population, lipid metabolism is altered, and higher levels of plasma triglycerides (TG) and cholesterol are seen in the older population. This can cause various health problems including cardiovascular disease and diabetes (Chappus-McCendie et al., 2019). In older individuals the brain goes through multiple morphological changes. With aging, there is a reduction in the total brain volume and grey matter volume. More specifically, grey matter volume in the cortex, cerebellum, frontal lobe, limbic lobe, temporal lobe, and parietal lobe is reduced. Additionally, white matter in the cortex and cerebellum also decreases with age (Chappus-McCendie et al., 2019). Atrophy of the hippocampus is a characteristic for Alzheimer's disease (Jahn, 2013). A study investigated how the hippocampus plays a role in cognitive aging and found the reduction in the hippocampus volume was associated to cognitive decline in episodic memory, working memory,

processing speed and executive function (O'Shea et al., 2016). A meta-analysis with 28 studies looked at the atrophy rate of the hippocampus in healthy individuals aging. They found the atrophy rate of the hippocampus to be 0.85% per year. When studies are categorized by mean age groups, studies with the average age less than 55 years has 0.38% atrophy per year, studies with mean age between 55 and 70 years have an atrophy of 0.98% per year, and studies with average age greater than 70 years has an atrophy of 1.12%. They also showed age was linked to elevated rate of hippocampal atrophy (Fraser et al., 2015). Moreover, older age groups have a higher prevalence of having Alzheimer's disease. Around ten percent of people between the ages of 65 and 74 years, and 32% of individuals above 80 years of age developed Alzheimer's disease in United States (Askarova et al., 2020). A prospective, longitudinal study with 128 participants found brain changes related to Alzheimer's disease to begin 15 years prior to the onset of expected symptoms. The onset of expected symptoms for the participants used in this study was the age the symptoms of their parent started. Twenty-five years prior to the onset of expected symptoms, there was a decrease in amyloid-β in the cerebrospinal fluid. Fifteen years prior to the onset of expected symptoms, there was amyloid-β deposits, brain atrophy and higher tau protein levels in the cerebrospinal fluid (Bateman et al., 2012). Based on this, changes in the brain can occur prior to the onset of symptoms.

1.1.2 Genetics - Apolipoprotein E epsilon 4 allele (APOE4)

Alzheimer's disease can be classified as early onset or late onset. First symptoms of Alzheimer's disease presents between the ages of thirty to sixty-five, they are usually diagnosed as early-onset. When the first symptoms are presented after the age of sixty-five, AD patients are usually diagnosed with late-onset (Cacace et al., 2016)

Early-onset cases are usually related to gene mutations. As reviewed by Cacace et al., mutations in the genes to produce amyloid precursor protein (APP), presenilins 1 (PSEN1) and presenilins 2 (PSEN2) have augmented penetrance for early onset AD (Cacace et al., 2016). Among the individuals who have early onset Alzheimer's disease, less than one percent had APP mutations, six percent had PSEN1 mutations and one percent had PSEN2 mutations (Cacace et al., 2016). These mutations are normally passed on from a parent

and is autosomal dominant (Cacace et al., 2016), meaning it overrides the other alleles(variants) of the gene. Mutations in the PSEN1 gene has 100% penetrance (Pilotto et al., 2013), meaning all individuals who carry this autosomal dominant mutation will develop Alzheimer's disease. Symptoms in individuals with this mutation can first appear between the ages of thirty and fifty years. It is suggested the penetrance of PSEN2 mutation is not complete (Pilotto et al., 2013), meaning not everyone with mutations in PSEN2 develops Alzheimer's disease. The first symptoms can appear between the ages of 40 and 70 years for individuals with this mutation (Cacace et al., 2016) suggesting that there might be other factors such as environmental or other genetic predisposition that will increase the risk of developing AD or not in the population carrying this mutation.

Carrying the apolipoprotein E epsilon 4 allele is the main genetic risk factor for developing late-onset Alzheimer's disease (Conway et al., 2014). APOE4 allele can also increase the risk of developing early onset (Cacace et al., 2016). Sixty-five to seventy-five percent of patients with sporadic Alzheimer's disease are carriers of the APOE4 allele (Crean et al., 2011). The apolipoprotein E gene found on chromosome 19 has many single nucleotide polymorphisms, where the frequent ones alters the amino acid at position 112 and 158 (Husain et al., 2021). The APOE gene has three isoforms in humans: epsilon 2 (APOE2); epsilon 3 (APOE3); and epsilon 4. In apoE2 isoform, amino acid at position 112 and 158 are cysteine. In apoE3 isoform, amino acid at position 112 is cysteine and at 158 is arginine. In apoE4, the amino acid at position 112 and 158 are arginine (Weisgraber et al., 1981).

Most common allele is the epsilon 3, where globally 79% of the population has it. Seven percent of the population has the epsilon 2 and 14% has the epsilon 4 allele (ALZGENE, 2010). APOE3 is considered as the normal isoforms, as APOE4 is associated with increased risk of Alzheimer's disease and APOE2 is associated with hyperlipoproteinemia (as reviewed by Y. Huang & Mahley, 2014). A meta-analysis found in the Caucasian population, individuals who are heterozygous for APOE4 (APOE2/APOE4 or APOE3/APOE4) have 2 to 3 times greater risk of developing Alzheimer's disease. Caucasians who are homozygous for APOE4 have 12 to 15 times greater risk of developing

Alzheimer's disease (Farrer et al., 1997). ApoE is synthesized by different tissues within the body, predominantly the liver followed by the brain. It can also be synthesized in the adrenal glands, testis, ovaries, skin, spleen, adipose tissue, lungs, kidneys, macrophages and "retinal pigment epithelial cells" (as reviewed by Huang & Mahley, 2014; Minihane et al., 2007). It has an important role in transporting lipids in the plasma and different tissues in the body, including the brain. When there is nerve damage in the peripheral, apoE production is stimulated, increasing apoE levels in the cerebral spinal fluid so lipid can be brought to areas that need to be restored. ApoE is a ligand that can bind to cell surface receptors that will trigger the uptake of fatty acids into the cells (Huang & Mahley, 2014). Individuals who carry at least one copy of the epsilon 4 allele would be referred to as carriers and individuals who do not carry the epsilon 4 allele will be referred to as non-carriers. The differences in the apoE isoforms structure and functions will be elaborated in section 1.3.

1.1.3 Environmental factors and Alzheimer's

Even though age and genetics are risk factors that cannot be changed, there are some factors that can be modified to reduce the risk of developing cognitive impairment. Some risk factors that can be modified includes unhealthy diet (Askarova et al., 2020), cigarette smoking, diabetes, hypertension, alcohol consumption (Durazzo, et al., 2014), obesity (Profenno, et al., 2010), , low level of education (Lindsay et al., 2002), and physical inactivity (Askarova et al., 2020). For optimal brain function, the brain needs a continuous supply of energy sources from the peripheral blood circulation (Berg, et al., 2002). Therefore, many cardiovascular risk factors such as diabetes, hypertension, obesity, high cholesterol, smoking and alcohol consumption also increases the risk of developing dementia (Whitmer, et al., 2005). Cigarette smoking disrupts the optimal vascular function due to the increased resistance of blood flow through the vessel (Ambrose & Barua, 2004). Therefore, cessation of smoking cigarettes can decrease the risk of developing Alzheimer's. A study found, individuals who were underweight or obese had a greater risk of developing Alzheimer's disease compared to individuals with a normal weight or overweight (Profenno et al., 2010). A meta-analysis found type II diabetes is a

risk factor to developing Alzheimer's disease with a relative risk of 1.1. When combining diabetes with the APOE4 genotype the risk increases to a relative risk of 5.5 (Peila et al., 2002). There are some lifestyle interventions that can decrease the risk of developing Alzheimer's disease. Being involved in mentally stimulating activities and having a high level of education can reduce the risk of developing Alzheimer's disease (Geerlings, et al., 1999; Lindsay et al., 2002). Individuals who have more education or a job that is mentally stimulating have more neural network (Geerlings et al., 1999). When neurons begins to degenerate due to aging, increased tau proteins hyperphosphorylation or amyloid beta plaques, they will be able to efficiently communicate with other neurons using different synapses (Geerlings et al., 1999). A meta-analysis reported that physical training was beneficial for cognitive abilities for the general population and in patients with Alzheimer's disease (Groot et al., 2016). Adopting a healthy diet can help prevent the risk of developing cognitive impairment. Additionally, there are increasing evidence from prospective studies supporting a role between consuming a diet rich in fatty fish containing omega-3 fatty and lower risk of developing dementia (Devore et al., 2009) and Alzheimer's disease (Pontifex et al., 2018).

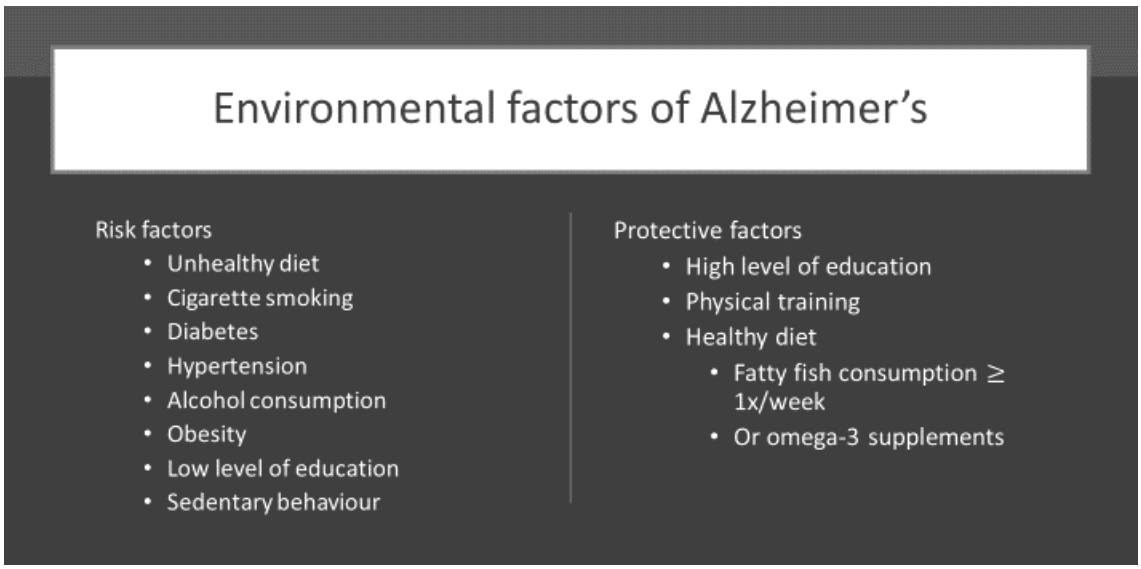

Figure 1 Environmental factors that can increase or sdecrease the risk of developing Alzheimer's disease.

1.1.3.1 Fish consumption and Alzheimer's disease

A few longitudinal studies have reported that regular fatty fish consumption decreases the risk of developing Alzheimer's disease. In the Rotterdam study, food consumption of 5,386 cognitively normal participants were assessed at baseline and at a follow-up about 2.1 years later. Cognitive tests were done to examine for dementia. They demonstrated consumption of more than 18.5 g of fish daily reduced the risk for developing dementia (RR = 0.4) and Alzheimer's disease (RR = 0.3) compared to consuming less than 3 g of fish daily (RR = 1.0) (Kalmijn et al., 1997). In a cohort with 1674 individuals above the age of 65 years with at least one follow-up within seven years, another group found that consuming fish or seafood at least once a week significantly decreased the risk of developing dementia compared to those who ate fish or seafood less than once per week ($p = 0.0091$) (Barberger-Gateau et al., 2002). Another longitudinal study had 815 participants aged between 65 and 94 years who were not diagnosed with Alzheimer's disease at baseline fill up a dietary questionnaire and had an average follow-up of 3.9 years. They found that participants who consumed fish at least once a week had 60% less risk of developing Alzheimer's disease when related to participants that hardly or never consumed fish (Morris et al., 2003). In a longitudinal study by Huang et al. with 2233 participants had an average follow-up of 5.4 years. They compared the risk of developing dementia and Alzheimer's disease in those who consumed fish at least twice a week and those who consumed fish less than once a month. They also divided the group based on whether or not the participant carried the APOE4 allele. Non-carriers of the APOE4 alleles who consumed fish at least twice a week had 28% less risk of developing dementia and 41% less risk of developing Alzheimer's disease. In APOE4 carriers, there was no reduced risk of developing dementia nor Alzheimer's disease when fish was consumed at least twice a week (Huang et al., 2005). Therefore, consumption of fish at least once a week on a regular basis decreases the risk of developing dementia and Alzheimer's disease in non-carriers of the APOE4 allele.

1.1.3.2 Omega-3 fatty acids and cognitive impairment

In an epidemiology study (InCHIANTI) by Cherubini and colleagues selected participants who were 65 and older (Cherubini et al., 2007). They demonstrated individuals who developed dementia had lower omega-3 fatty acids levels in the plasma compared to participants who did not have cognitive impairment (Cherubini et al., 2007). Data from this study was adjusted for potential confounders such as "age, gender, education, smoking status, BMI, weight loss, alcohol intake, total energy intake, low density lipoprotein [LDL] and high density lipoprotein [HDL] cholesterol levels, triglyceride levels, [coronary heart disease], [cerebrovascular disease], [congestive heart failure], diabetes, hypertension, and depression" (Cherubini et al., 2007). Furthermore, Conquer and colleagues demonstrated individuals with Alzheimer's disease had lower docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and total omega-3 fatty acids levels in the plasma total phospholipid and phosphatidylcholine (PC), and phosphatidylethanolamine (PE) compartments compared to elderly participants with normal cognitive ability (Conquer et al., 2000). However, their groups were not matched based on age and individuals in the Alzheimer's group were significantly older than the cognitively normal group (Juli A. Conquer, et al., 2000).

Consuming omega-3 fatty acids, such as EPA and DHA can have many beneficial effects including prevention for Alzheimer's disease (Boudrault et al., 2009; Cunnane et al., 2012; Mozaffarian & Wu, 2012; Pontifex et al., 2018; Skulas-Ray et al., 2011). From epidemiological studies, there has been several randomized, double-blind, placebo control clinical trials evaluating if a supplementation could prevent Alzheimer's disease onset. A study had cognitively healthy older adults (n = 867) with a mean age of 75 years take omega-3 fatty acid supplements (200 mg of EPA and 500 mg of DHA per day) or placebo supplements daily for 24 months. They were testing whether taking omega-3 fatty acid supplements would benefit the cognitive ability of participants (Dangour et al., 2010). Serum EPA and DHA levels were higher in individuals who took omega-3 supplements than individuals who took the placebo supplements for 24 months. However, there were no differences in cognitive abilities between the individuals who

consumed omega-3 fatty acid supplements and those who took the placebo supplements. Both groups did not have cognitive decline throughout the study and can be explained by the short duration of the intervention (Dangour et al., 2010). Furthermore, some investigators tested whether omega-3 supplementation could benefit those who already have cognitive impairment or Alzheimer's disease. A study assessed the effects of Alzheimer's patients (n= 174) consuming omega-3 fatty acid supplements for six months to their cognitive functions. There was no difference in the rate of cognitive decline compared to control group after consuming omega-3 fatty acid supplements (1.7 g of DHA and 0.6 g of EPA) everyday for six months. However, in a small subgroup of patients with very mild Alzheimer's disease no cognitive decline was observed from baseline and six months, where in the control group with very mild Alzheimer's disease a cognitive decline was observed (Freund-Levi et al., 2006). Another randomized, double-blind controlled study was conducted by Philip and colleagues (Phillips, et al., 2015). Fifty-seven participants with cognitive impairment, but not dementia and nineteen participants with Alzheimer's disease were randomized into the omega-3 or placebo supplement groups. The omega-3 fatty acids (600 g of EPA and 625 mg of DHA) or placebo supplements were taken every day for four months. They found consuming omega-3 fatty acid supplements did not benefit those with cognitive impairment and with dementia (Phillips et al., 2015). A review article elucidates how observational studies looking at the role of APOE status on the association of omega-3 fatty acids and cognitive outcomes are inconsistent. Some studies have shown there was no effect of APOE genotype on the omega-3 fatty acids link to cognitive functions. Some studies observed a cognitive benefit in only non-carriers of the APOE4 allele who consumed omega-3 fatty acids. Other studies showed only APOE4 carriers benefitted cognitively with omega-3 fatty acids. However, the studies that found positive association of omega-3 fatty acids to cognitive function were in younger participants (Yassine, et al., 2017). Therefore, they concluded consumption of omega-3 fatty acids as a preventative approach (predementia) in APOE4 carriers can reduce their risk of developing the disease or delay the onset of the disease (Yassine, et al., 2017).

Before going in further details about the importance of omega-3 fatty acids for neuronal health, lipids and its metabolism will generally be elucidated.

1.2 Lipids

Lipids are one of the three main macronutrients found in the diet. The other two are carbohydrates and proteins. One of the main characteristics of lipids that differentiates it from carbohydrates and proteins is that it is hydrophobic, non-polar and composed of mostly hydrocarbon chains. Lipids has various functions throughout the body, which includes composition of the membrane, energy storage and precursors of metabolites (Gibney et al., 2009; Iqbal & Hussain, 2009). Lipids are fatty acids that can be esterified to alcohol (glycerol), or cholesterol and/or other groups. Fatty acids are hydrocarbon chain with a carboxylic acid terminal. They can be of various lengths and degrees of saturation and unsaturation (Gibney et al., 2009). In animals, the fatty acid chain length normally varies from C14 to C24. They can also be characterized by its degree of saturation or unsaturation which is based on the number of double bonds present in its structure. Saturated fatty acids have no double bonds present in their hydrocarbon chain. Monounsaturated fatty acids have one double bond present, forming a kink in its structure. Polyunsaturated fatty acids have more than one double bond in its hydrocarbon chain (Gibney et al., 2009). The fatty acids can be further characterized based on where the first double bond from its methyl terminal is located. If the first double bond from the methyl terminal is located on the third carbon (between the third and fourth), the fatty acid would be referred to as omega-3 fatty acid. For omega-6 fatty acids, the first double bond in the hydrocarbon chain from the methyl terminal would be on the sixth carbon (between sixth and seventh). A good source of omega-3 fatty acids can be found in fatty cold-water fish like salmon, mackerel, sardines and fresh tuna, and in flaxseed oil, canola oil and peanut oils (Chang et al., 2009; Fisk et al., 2018).

1.2.1 Lipid Compartments

Fatty acids in the body can be esterified in many forms, and therefore be compartmentalized into different lipid compartments. Some lipid compartments include free fatty acids (FFA), triglycerides, cholesteryl esters (CE), and phospholipids (PLs). FFA

are non-esterified fatty acids, meaning the hydrocarbon chain is not esterified and has the carboxylic acid terminal. Triglycerides has three fatty acids esterified to a glycerol backbone. The three fatty acids can be of different length. CE is a fatty acid esterified to a cholesterol. Phospholipids has two fatty acids esterified to a glycerol backbone at the sn-1 and sn-2 positions, and a phosphate group esterified at the sn-3 position with an alcohol attached to the phosphate (Berg et al., 2002). Compared to the other lipid compartments PLs are amphiphilic, meaning it has a hydrophobic and hydrophilic component. The PL head makes it polar, and the fatty acids chains attached to the glycerol are non-polar. PLs can be further distinguished based on the different alcohol attached to the phospholipid head. That differentiation can separate PLs into more lipid compartments such as PC and PE, which are the most abundant PLs in animal tissue (Gibney et al., 2009). There is also a PL compartment called lyso-phosphatidylcholine (LPC). It has the same structure as PC, except it has only one fatty acid attached to the glycerol backbone instead of two. Predominant dietary fats are in the TG compartment (Iqbal & Hussain, 2009).

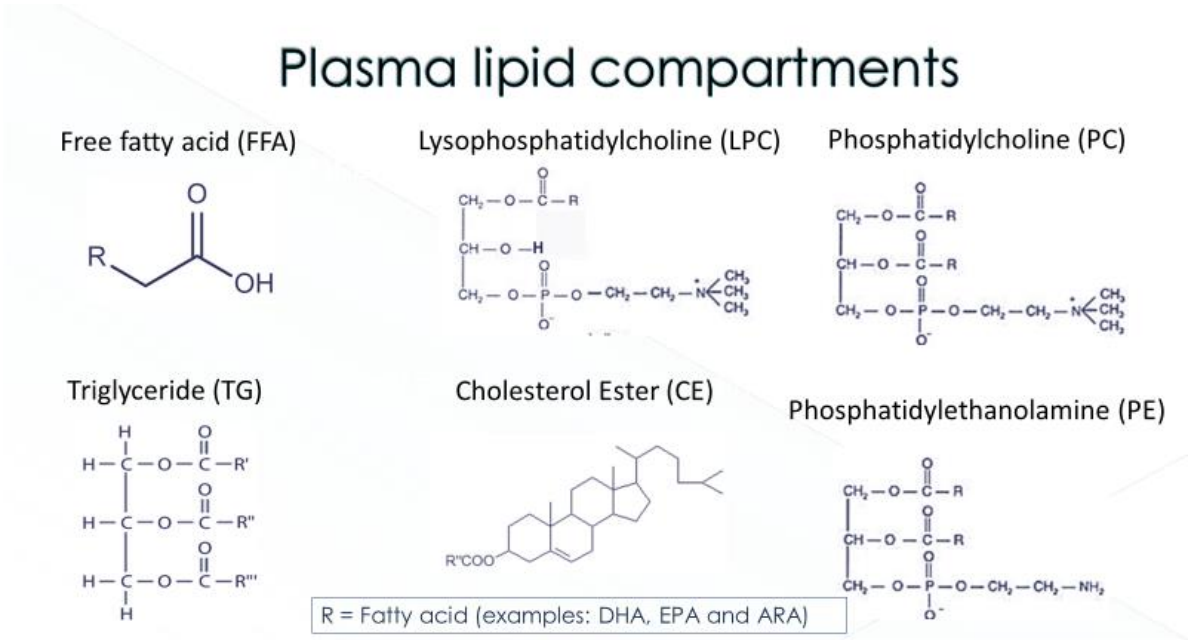

Figure 2 Some of the different plasma lipid compartments. FFA is non-esterified fatty acid. TG has three fatty acids esterified to a glycerol backbone. Lysophosphatidylcholine has one fatty acid esterified to a glycerol backbone with phosphate group and choline. Phosphatidylcholine is similar to lysophosphatidylcholine but has two fatty acids attached to a glycerol backbone with a phosphate group and choline. Phosphatidylethanolamine is similar to phosphatidylcholine, but instead of a choline attached to the phosphate group, an ethanolamine is attached to the phosphate group. Cholesterylester has one fatty acid esterified to a cholesterol. "R" in this figure represents a fatty acid. Examples of fatty acids are docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA)

1.2.2 Lipid digestion to absorption

Lipids from our diet are digested, absorbed, and transported to various cells throughout the body. Digestion of fats begins in the oral cavity. Lingual lipases are secreted from the palate it starts the hydrolysis of TG. Once it enters the stomach, lipases will further lipolyze the lipids. The stomach is mostly mechanical as it digests the food by churning and by this the dietary fats will emulsify to form chyme. As chyme enters the duodenum, it will be solubilized with bile salts and the dietary fat will be lipolyzed by lipase (Gibney et al., 2009). In the upper portion of the jejunum, TG will get hydrolyzed by pancreatic lipases at the sn-1 and sn-3 positions, producing two FFAs and one 2-monoacylglycerol. Monoacylglycerol can be further hydrolyzed by cholesterol esterase to produce one more FFA and glycerol. The most abundant PL in the intestine is PC. PC gets hydrolyzed by pancreatic phospholipase A2 mainly, where it releases a fatty acid at the sn-2 position and becomes LPC. Cholesterol esterase hydrolyzes CE to form a FFA and free cholesterol (Iqbal

& Hussain, 2009). With the help of transport proteins, all the products formed from the hydrolyses (FFA, LPC, monoacylglycerol, glycerol, and free cholesterol) can be absorbed by the intestinal cells. Intestinal cells are also known as enterocytes. Once the by-products enter the enterocyte, they will go through the cytoplasm towards the endoplasmic reticulum. In the endoplasmic reticulum, the lipids are resynthesized and packaged into chylomicrons. Chylomicrons are lipoproteins that will transport exogenous lipids throughout the body (Iqbal & Hussain, 2009). First, the chylomicrons will get exported out of the intestinal cell and into the lymphatic vessels. Then, they will enter the blood circulation at the left thoracic duct so that lipids can be distributed to cells (Gibney et al., 2009).

1.2.3 Lipoproteins

Lipoproteins transport lipids and cholesterol in the blood. There are four main lipoproteins found in the plasma: chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). Lower the density of a lipoprotein means the lipoprotein has more lipids compared to the protein level. All lipoproteins have a common structural feature. The outer surface is made with the hydrophilic molecules. This includes the polar phospholipid head groups, and the hydrophilic proteins like apolipoproteins. The hydrophobic molecules are in the inner core of the lipoprotein, such as TG, CE, phospholipid and lysophospholipid tails (fatty acid). FFA are not transported in lipoproteins in the plasma as they are transported bound to albumin (Lacombe et al., 2018). The main role of chylomicron and VLDL is to bring TG to the cells as they are the main energy source in lipids (Gibney et al., 2009; Iqbal & Hussain, 2009). The main role of LDL is to deliver cholesterol to cells (Berg, 2002a). HDL removes excess cholesterol from the peripheral tissues and brings it to the liver to be sent to the intestine to either be excreted out of the body or recycled (Berg et al., 2002a; Cohen, 2008). Lipids absorbed from the diet leave the intestine by exocytosis as chylomicrons, which is the largest lipoprotein and is mostly filled with TG (J. M. Berg et al., 2002a) .

In postprandial, the quantity of lipids present in the plasma is seven times higher than the fasting lipid levels (Iqbal & Hussain, 2009). As mentioned earlier, chylomicrons transport

exogenous dietary fats into the plasma. Around 99% of its content is TG (Berg et al., 2002a). In the blood circulation, apolipoproteins present on the surface of chylomicrons will bind to specific lipoprotein receptors and activate lipases (Gibney et al., 2009). Lipoprotein lipase is on endothelial cell surfaces of capillaries mainly in adipose tissues, skeletal muscles and heart (Mahley & Ji, 1999). The lipases will breakdown the lipoprotein and hydrolyze the TG so that the cells can take the lipid by-products where it is needed. The lipids that are not used by the cells are re-packaged into chylomicron remnants that are smaller and denser in lipids than chylomicrons and goes to the liver. In the liver, the chylomicron remnants becomes VLDL (Gibney et al., 2009). VLDL can also be directly produced by the liver with lipids that are synthesized by the liver (Feingold & Grunfeld, 2000). Similar to chylomicrons, the main function of VLDL is to transport TG to peripheral tissues. VLDL gets lipolyzed by lipases and by the time it returns to the liver, it becomes smaller and denser. The liver will convert it to LDL. As most of the TG are used up or stored, LDL is comparatively concentrated with cholesterol (Mahley & Ji, 1999). The main role of LDL is to transport cholesterol to cells. Having high serum cholesterol levels in LDL form part-takes in the development of atherosclerotic plaques in the arteries. Therefore, LDL cholesterol is considered as "bad cholesterol". HDL helps with the removal of excess cholesterol. Hence, cholesterol found in HDL is considered "good cholesterol". HDL binds to cholesterol released by cells and re-esterifies it to cholesteryl esters. Then CE is brought to the liver to be excreted out of the body or recycled to produce steroid hormones with cholesterol (Berg et al., 2002a).

1.3 The different apolipoprotein E isoforms and its function

Apolipoproteins has an important function in the metabolism of lipoproteins (Feingold & Grunfeld, 2000). There are different types of apolipoproteins. ApoE is found on the surfaces of lipoproteins such as chylomicrons, chylomicrons remnants, VLDL and certain subsection of HDL. It is a ligand for LDL receptor family. Apolipoprotein B-100 is found of the surfaces of VLDL and LDL and is also a ligand for LDL receptor (Feingold & Grunfeld, 2000). LDL receptor (LDLr) family includes eight receptors. All those receptors have different binding affinity for the apoE ligand and varies between the apoE isoforms.

APOE4 and APOE2 have high affinity for LDLr and LDLr-related receptor 1 (Husain et al., 2021). VLDL receptor binds to all apoE isoforms equally (Ruiz et al., 2005).

ApoE protein consist of 299 amino acid, weighing around 36 kDa with three sections. The N-terminal section of the protein has four helices, where one of them contains the region that binds to low density lipoprotein receptor. The C-terminal section of the protein contains the region that bind to lipids (allowing it to be part of a lipoprotein). Between the N- and C-terminals there is a hinge connecting both terminals (Husain et al., 2021). In a review by Huang and Mahley, it was stated the different apoE isoforms preferred different lipoproteins based on the amount of apoE present on the surface of the lipoproteins. ApoE4 favoured VLDL, compared to apoE3 and apoE2 favoured HDL (Huang & Mahley, 2014). In the APOE4 isoform, at position 112 there is an arginine, where in APOE3 and APOE2 there is a cysteine. This changes the section of the protein involved in binding to lipid and alters APOE4 preference to VLDL. (Husain et al., 2021).

In the central nervous system, apoE is secreted "by astrocytes, oligodendrocytes, pericytes, choroid plexus and neurons" (Husain et al., 2021). ApoE has a role in the transportation of cholesterol and lipids to neurons and glial cells (Husain et al., 2021). In neurodegenerative diseases like Alzheimer's disease, the brain cholesterol turnover is higher (Dietschy & Turley, 2001). In the hippocampus of individuals with Alzheimer's disease, the lipid content is altered (Mendis et al., 2016). In the hippocampus of rats, astrocytes had high levels of apoE, where neurons had 3.6 folds less levels of apoE expressed (Rapp et al., 2006). ApoE4 was not able to sufficiently incorporate cholesterol in the hippocampus neurons compared to apoE2 and apoE3 isoforms (Rapp et al., 2006). Therefore, in the brain of APOE4 carriers, the cholesterol metabolism is modified as there is more turnover and less is incorporated in the neurons of the hippocampus.

1.4 Lipid and brain

Fifty to sixty percent of the brain dry weight in human is lipids (Haag, 2003) and is predominantly composed of PL (Velasco & Tan, 2014). More than one third of that is polyunsaturated fatty acids, which mainly includes ARA and DHA. These fatty acids have

important functions in the brain, such as for neurodevelopment, neurogenesis, neuronal transmission, membrane permeability, and neuroinflammation (Chouinard-Watkins et al., 2017; Haag, 2003; Lacombe et al., 2018).

1.4.1 Docosahexaenoic acid (DHA)

In the brain, 40% of polyunsaturated fatty acids is DHA, which is also the most common omega-3 fatty acid in the brain (Lacombe et al., 2018). The brain can form DHA from alpha-linolenic acid (ALA), an essential fatty acid. However, based on an animal study only less than 0.2% of ALA taken up by the brain is converted to DHA (DeMar et al., 2005). Therefore, the brain depends predominantly on the blood circulation to provide DHA for the brain membrane renewal (Lacombe et al., 2018). An essential fatty acid cannot be formed *de novo* in humans and must therefore be provided in the diet. The liver can convert ALA to EPA then to DHA with the help of desaturases and elongase (Haag, 2003; Lacombe et al., 2018; Plourde & Cunnane, 2007). EPA is also a common omega-3 fatty acid seen in the plasma. However, the rate of conversion of ALA to EPA is less than 5% and ALA to DHA is 0.5% (Plourde & Cunnane, 2007). For this reason, it is important to obtain DHA from our diet.

More than 80% of brain DHA is in the PL compartment, mainly in the PC and PE compartment. Mediated by phospholipase A2 (PLA2), DHA can be non-esterified and be converted into a metabolite involved in many cellular functions, especially regulating inflammation (Lacombe et al., 2018). EPA and DHA produces metabolites like resolvins which are involved in anti-inflammation and immunoregulation (Serhan, et al., 2008). A study that compared DHA content in 12 different regions in the central nervous system found DHA levels are much higher in the frontal cortex compared to regions like the spinal cord and brain stem with more myelination (Naudí et al., 2017). Frontal cortex is involved in high cognitive abilities, such as "working memory and social emotional evaluation of stimuli" (Felleman, 2009) which are affected in individuals with Alzheimer's disease (Jahn, 2013).

1.4.2 Arachidonic acid (ARA)

ARA is the most abundant polyunsaturated fatty acid in the brain (Haag, 2003). Like DHA, ARA cannot be synthesized *de novo,* but it can be synthesized from the conversion of linoleic acid, also an omega-6 fatty acid. Synthesis of ARA requires the same desaturases and elongase used to convert ALA into EPA (Haag, 2003; Plourde & Cunnane, 2007). Non-esterified ARA mediates in the production of pro-inflammatory metabolites like leukotrienes and prostaglandins (Sanchez-Mejia & Mucke, 2010). Metabolism of ARA can form prostaglandin using cyclooxygenase, which is concentrated in the hippocampus. Cyclooxygenase decreases long term potentiation, meaning it reduces signal transmission between two neurons and it affects spatial learning. ARA metabolism produces "reactive oxygen species" which creates oxidative stress. Increased levels of oxidative stress is seen in the brains of Alzheimer's disease patients (Sanchez-Mejia & Mucke, 2010).

Both DHA and ARA are found in the brain membrane in the PL compartment. Studies done *in vitro* observed DHA and ARA are metabolized differently. Both these fatty acids need to be non-esterified from the PLs using the PLA2 enzymes to be converted to active biomolecules. However, the release of DHA and ARA is done by different PLA2 enzymes. DHA is hydrolyzed at the sn-2 position by Ca^{2+} independent PLA2 and ARA is hydrolyzed by Ca^{2+} dependent cytosolic PLA2 (Lacombe et al., 2018). DHA and ARA compete to be bound to PL. If more DHA is consumed, there will be less ARA bound in PL (Sanchez-Mejia & Mucke, 2010). This allows less ARA to be released by PLA2 during hydrolysis (Sanchez-Mejia & Mucke, 2010) to be converted to pro-inflammatory metabolites that can induce

apoptosis.

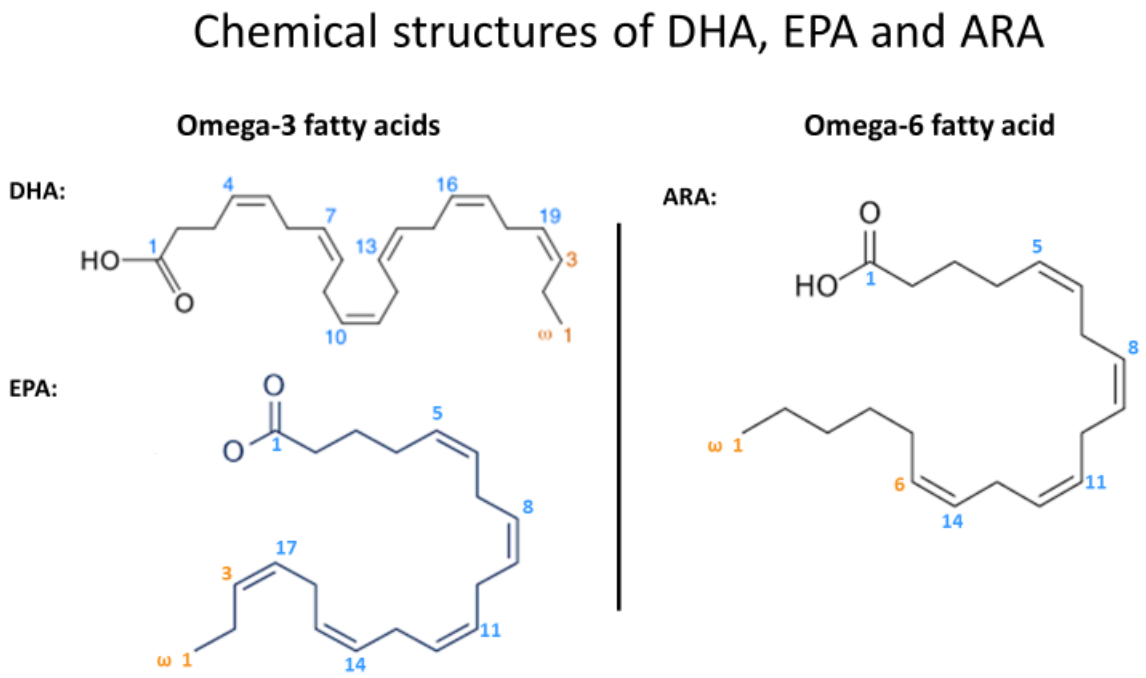

Figure 3 Chemical structures of DHA, EPA and ARA. DHA and EPA are omega-3 fatty acids. The first double bond from its methyl (ω) terminal is on the third carbon. Chemical formula of DHA is $C_{22}H_{32}O_2$ and EPA is $C_{20}H_{30}O_2$. ARA is an omega-6 fatty acid, where the first double bond from its methyl (ω) terminal is on the sixth carbon. Chemical formula of ARA is $C_{20}H_{32}O_2$ DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid.

1.4.3 Brain uptake of fatty acids

The brain can take-in fatty acids in the non-esterified and LPC form from the plasma. In the plasma FFA and LPC are transported predominantly bound to albumin. Thirty percent of DHA in the LPC compartment is transported in HDL and LDL in the plasma. Non esterified DHA has a half-life of about thirty seconds and in LPC of about five to ten minutes, meaning DHA in these compartments are quickly utilized by cells (Lacombe et al., 2018). Before the brain uptake of DHA in the non-esterified and LPC compartment, DHA needs to be released from albumin and lipoproteins. Lipoproteins that transport fatty acids can be hydrolyzed by lipases found at the surface of the brain endothelium and release FFA and LPC. FFA can enter the endothelium of the blood brain barriers by passive diffusion. DHA in the LPC compartment goes across the endothelial membrane potentially mediated by the transporter major facilitator superfamily domain containing 2A (Mfsd2a).

The endothelium of the blood brain barrier is rich in Mfsd2a. Once DHA exits the plasma membrane, it binds to a protein that will help transport it through the cytosol and to the basal membrane to then enter the brain by passive diffusion. In the brain DHA in the FFA form is converted first to DHA-CoA by fatty acid transport protein or long-chain fatty acid acyl-CoA synthetase. DHA-CoA is converted to phosphatic acid by esterifying to lysophosphatidic acid. Phosphatidic acid can be converted to PE, PC, or phosphatidylinositol. More than 80% of DHA in the brain is esterified to PL, where PE and PC are the most abundant. Mediated by the enzyme PLA2, polyunsaturated fatty acids will be hydrolyzed at the sn-2 position to either be converted to bioactive metabolites, beta-oxidized or re-modelled into a PLs again (Lacombe et al., 2018) as seen in an animal study. Chouinard-Watkins et al. intravenously gave four mice C14 radiolabelled DHA in the FFA or LPC form (Chouinard-Watkins et al., 2017). The brain uptake of DHA in the FFA compartment was higher than in the LPC compartment. Additionally, they found once DHA enters the brain it gets remodelled into different PL compartments, predominantly in the PE and followed by PC compartment (Chouinard-Watkins et al., 2017) and can then be incorporated in the brain membrane. Thus overall, DHA can be taken up by the brain in the non-esterified form or esterified to LPC, and once it enters the brain it can be metabolised right away or stored in membrane PLs.

1.5 Omega-3 fatty acid metabolism disrupted in APOE4 carriers

The first evidence our team obtained for the disruption in the metabolism of omega-3 fatty acids in APOE4 carriers was in a study published by Plourde et al. (Plourde, et al., 2009). This study quantified the amount of EPA and DHA present in the plasma PL, TG, FFA and CE before and after omega-3 fatty acid supplementation (1.9 g/day of EPA and 1.1 g/day of DHA) for six weeks and compared it between APOE4 carriers (n=8) and non-carriers (n=20). Pre-supplementation of omega-3 fatty acids, APOE4 carriers had 67% higher EPA and 60% higher DHA levels in TG compared to non-carriers. Additionally, post-supplementation DHA levels in TG increased by 65% in APOE4 carriers and by 180% in non-carriers and a gene by diet interaction was seen for DHA in TG. A gene by diet interaction was also seen for EPA in FFA. However, no differences were seen pre-

supplementation or post-supplementation for EPA and DHA in PL and CE compartments. Based on these findings, it seems the metabolism of DHA and EPA in TG was less efficient in APOE4 carriers (Plourde et al., 2009). Another study looked at the DHA metabolism by administering C13 labelled DHA. They gave a single oral dose of 40 mg of C13 DHA and evaluated the metabolism of it for a 28 days period in APOE4 carriers (n=6) and non-carriers (n=32) (Chouinard-Watkins et al., 2013). Mean plasma concentration in APOE4 carriers was 31% less compared to non-carriers over the 28 days ($p = 0.04$). Additionally, they quantified the amount of 13C-DHA that was beta-oxidized by the amount of 13C-CO2 exhaled. After one cycle of beta-oxidation, 13C-CO2 will be produced and then exhaled. Based on the amount of C13-CO2 exhaled, they found DHA was beta-oxidized more in APOE4 carriers ($p = 0.003$) (Chouinard-Watkins et al., 2013), suggesting DHA is degraded to produce energy more than packaged into PL in APOE4 carriers compared to non-carriers. Another study examined the ARA to DHA ratio in 195 cognitively normal individuals. Within three years after the collection of their blood samples, 12% of them developed mild cognitive impairment or Alzheimer's disease. They found an association between APOE4 carriers who developed a diagnosis and having a high ARA to DHA ratio in PC ($p = 0.001$), LPC ($p < 0.001$), PE ($p = 0.027$) and phosphatidylinositol ($p = 0.003$) compared to cognitively normal APOE4 carriers and non-carriers. The author suggested that an increase in the ARA to DHA ratio in the PL compartment can be a potential biomarker for pre-clinical mild cognitive impairment and Alzheimer's disease with the APOE4 genotype (Abdullah et al., 2017).

1.5.1 DHA uptake by the brain is altered in APOE4 carriers

Fatty acid profiles in human brains are not easily accessible for ethical reasons, so animal models are used to understand the brain uptake of DHA. However, a recent study by Yassine and his colleagues looked at the rate [1-11C]-DHA was incorporated in different brain regions using positron emission tomography, often abbreviated as PET in 22 healthy adults with an average age of 35 years. To their knowledge, they are the first to use this technique to observe the incorporation of DHA in APOE4 individuals (Yassine, et al., 2017). They found the mean DHA incorporation rate of the radio labelled DHA in global

grey matter was 16% higher in APOE4 carriers compared to non-carriers. They additionally found DHA was incorporated at a higher rate in many brain regions, especially in the entorhinal subregion. This region is affected in the earlier stages of Alzheimer's disease progression. When they looked at the DHA incorporation rate in the whole brain, there was no difference between APOE4 carriers and non-carriers. They suggested a potential explanation for the higher DHA incorporation seen in APOE4 carriers is that there is more DHA loss in their brain (Yassine, et al., 2017). Furthermore, an animal study done in mice homozygous for the APOE4, APOE3 or APOE2 looked at the brain uptake of DHA at four months and thirteen months of age. APOE4 mice had lower levels of DHA in the brain at four months compared to APOE2 mice and at thirteen months compared to both APOE2 and APOE3 mice (Vandal et al., 2014). However, they also measured the cerebrovascular volume and found the volume was similar among the different APOE genotypes (Vandal et al., 2014). Therefore, the number of blood vessels present in the brain is not the reason behind the lower uptake of DHA in APOE4 carriers. As mentioned earlier, the brain relies on the peripheral circulation for DHA uptake, so these results suggest the transportation of DHA to the brain can be affected in APOE4 carriers.

2. Rationale and hypothesis

Alzheimer's disease is a neurodegenerative disease that affects memory, language skills, judgement, and eventually ability to do everyday tasks like walking and swallowing. Currently there is no treatment nor preventative approach for it. Studies have found consuming omega-3 fatty acids (DHA and EPA) is associated with reduced risk of developing the disease. In some studies, when stratified by APOE4 genotype, beneficial effect is not seen in individuals who carry the APOE4 allele. We do not know the mechanisms on how omega-3 fatty acids can benefit cognitive functions, but we know the brain contains a lot of DHA and depends on the peripheral circulation for DHA. Studies have shown metabolism of omega-3 fatty acids is disrupted in APOE4 carriers. ApoE protein is involved in the transportation of lipids in the blood and brain, and the removal of excess lipids found in the blood. The brain can take-in DHA in the FFA and LPC blood compartment. Therefore, we hypothesize in APOE4 carriers there is a compartment

packaging issue of DHA. More precisely, after supplementation with omega-3 fatty acids for six months, the concentration of DHA and EPA will not rise in APOE4 carriers compared to non-carriers in FFA and LPC, the lipid compartments the brain can take-in fatty acids, and that DHA is packaged more in TG compartment in APOE4 carriers.

2.1 Objectives

Primary objective of this study is to compare plasma DHA, EPA and ARA concentrations in the different lipid compartments (LPC, PC, PE, FFA, TG and CE) based on omega-3 supplementation (pre versus post-supplementation) and APOE genotype (APOE4 non-carrier versus carrier). Secondary objective of this study is to compare ΔDHA, ΔEPA and ΔARA concentrations (Δ = post – pre) in the different lipid compartments of the plasma based on BMI (low versus high BMI) and APOE genotype (APOE4 non-carrier versus carrier).

3. Material and Methods

3.1 Participant recruitment criteria and plasma collection

The current study is an add-on study to a randomized controlled trial conducted by the laboratory group of Pr. Mélanie Plourde. Interested participants went through a screening process. Inclusion criteria included males and females between the ages 20 and 80 years. Exclusion criteria included: diabetes diagnosis within last 6 months; cancer diagnosis within last 6 months; uncontrolled hypothyroidism or hyperthyroidism; smoking; alcoholism; autoimmune disorders such as rheumatoid arthritis and multiple sclerosis; uncorrected visual impairments; native language is not French; cognitive disorders; brain injuries; mental health disorders that can influence cognitive functions; consumption of omega-3 fatty acid supplements within 6 months to recruitment; more than 2 portions of fatty fish regularly consumed a week; and intense physical workout. Any medication participants were taking had to be stable for at least 6 months prior to recruitment. Additionally, participants' blood samples were analyzed to ensure blood TG ≤ 1.7 mmol/L, HDL-cholesterol ≥ 1.3 mmol/L, glucose < 6 mmol/L, TRH < 3.5 mUI/L, C-reactive protein <10 mg/L, and white blood cell count 4000-11000 µL.

Eligible participants were randomized into the placebo (corn / soybean oil) or omega-3 fatty acid (1.4 g of DHA and 1.8 g of EPA) supplement groups. The supplementation was taken everyday for six months. In the current add-on study, we analysed the plasma samples from the omega-3 fatty acid supplement group only. Blood samples were collected prior to starting the intake of the supplements and once every month as they were taking the supplements up to six months. After collection, blood was centrifuged at 3400 rpm for 10 minutes at 4°C. The plasma found at the top phase was aliquoted and stored in a -80 °C freezer until further analysis. For the current project, the plasma samples taken prior to the start of taking the supplements (pre-supplementation) and plasma samples taken after six months under the supplement (post-supplementation) were analyzed. Only plasma samples of APOE4 carriers and non-carriers matched based

on sex, age ± 5 years, and body mass index (BMI) ± 5.3 kg/m^2 of the participants were analysed.

3.2 APOE4 genotyping

Apolipoprotein E status was determined using predesigned TaqMan SNP genotyping assays (Applied Biosystems, Foster city, United States) for rs429358 and rs7412 (SNP ID) at the RNomics Platform (Centre de recherche du CHUS, Sherbrooke, Canada). To do this, the DNA was first extracted using the QIAmp DNA extraction kit (Qiagen, Hilden, Germany) from 200 µL of thawed whole blood following the manufacturer's protocol and then genotyped using the assays mentioned earlier. DNA fragment with the APOE gene was amplified by PCR using the Stratagene Mx3005P multiplex quantitative system (Agilent Technologies, La Jolla, United States). In this, the samples went through 40 cycles of amplification, where each cycle involved heating the double strand DNA for 30 sec at 95 °C to separate the strands and 1 min at 60 °C for the primer/probe mix to anneal to the target DNA region and amplify the target DNA sequence. The APOE status of each participant was determined and then separated in two groups depending on if they carry one or two copies of the APOE4 allele (APOE4 carriers) or not carrying any copy (non-carriers).

3.3 Lipid extraction and separation from the plasma samples

3.3.1 Extraction of lipids from the plasma samples

The plasma contains a mixture of fatty acids esterified in different ways and in this project, we have focussed on CE, TG, FFA, PE, PC and LPC. Total lipids were first extracted from the plasma using the Folch et al. method (1957). This was done by using 500 µL of the plasma and adding internal standards for each lipid compartment that will be analyzed as follows: 0.117 mg of C17 CE (Lot no CH-816-S12-B, NU-CHEK PREP, INC), 0.0615 mg of C17 TG (Lot no T-155-AU14-B, NU-CHEK PREP, INC), 0.0105 mg of C15 FFA (Lot no SHBL3424, Sigma Life Sciences), 0.0054 mg of C17 PE (Lot no 830756-01-020, Avanti polar lipids, INC), 0.084 mg of C15 PC (Lot no 850350P-500MG-A-038, Avanti polar lipids, INC), and 0.0045 mg of C17 LPC (Lot no 855676P-25MG-B-033, Avanti polar lipids, INC). More precisely, 3 mg of each internal standard was weighed individually in 10 mL

volumetric flasks. Internal standards for CE, TG, PE, PC and LPC were dissolved in 10 mL of 2:1 chloroform methanol and FFA was dissolved in 10 mL of chloroform only. Precise volumes of the internal standard solutions were added to the plasma sample to reach the quantity written above: 390 µL of CE; 205 µL of TG; 35 µL of FFA; 18 µL of PE; 280 µL of PC; and 15 µL of LPC. 10 mL of 2:1 chloroform methanol was added to the sample. The solution was shaken and placed in the dark for one hour. Then 2 mL of a solution of water with NaCl (0.9%) was added to help separate the chloroform phase which contained the lipids from the aqueous phase. The chloroform phase with the lipids were collected in a separate test tube and chloroform was evaporated under nitrogen stream. Once the lipids from the plasma were extracted the first thin layer chromatography (TLC) was performed to proceed in a first lipid class separation.

3.3.2 Separation of PL, TG, CE and FFA by thin layer chromatography

To separate CE, TG, FFA and total PLs, a first thin layer chromatography was performed. Dried total lipids extracted from the plasma (as described above) were reconstituted in 50 µL of chloroform. The TLC plates used were 20 x 20 cm with 250 µm thickness of silica with a F-254 indicator from SiliCycle (SiliCycle, Quebec city, Canada). Before loading the samples onto the TLC plates, the plates were heated for one hour at 110°C to remove trace of water within the silica gel and activate the plates. On each plate, five samples were loaded at two cm from the bottom of the plate and samples were deposited in bands of three-cm wide as illustrated in Figure 3.1. A mix of external standards was loaded in the last lane to confirm the lipid separation. Once the plate was loaded with the samples, it was placed into a TLC glass chamber where a mixture of solvent was added.

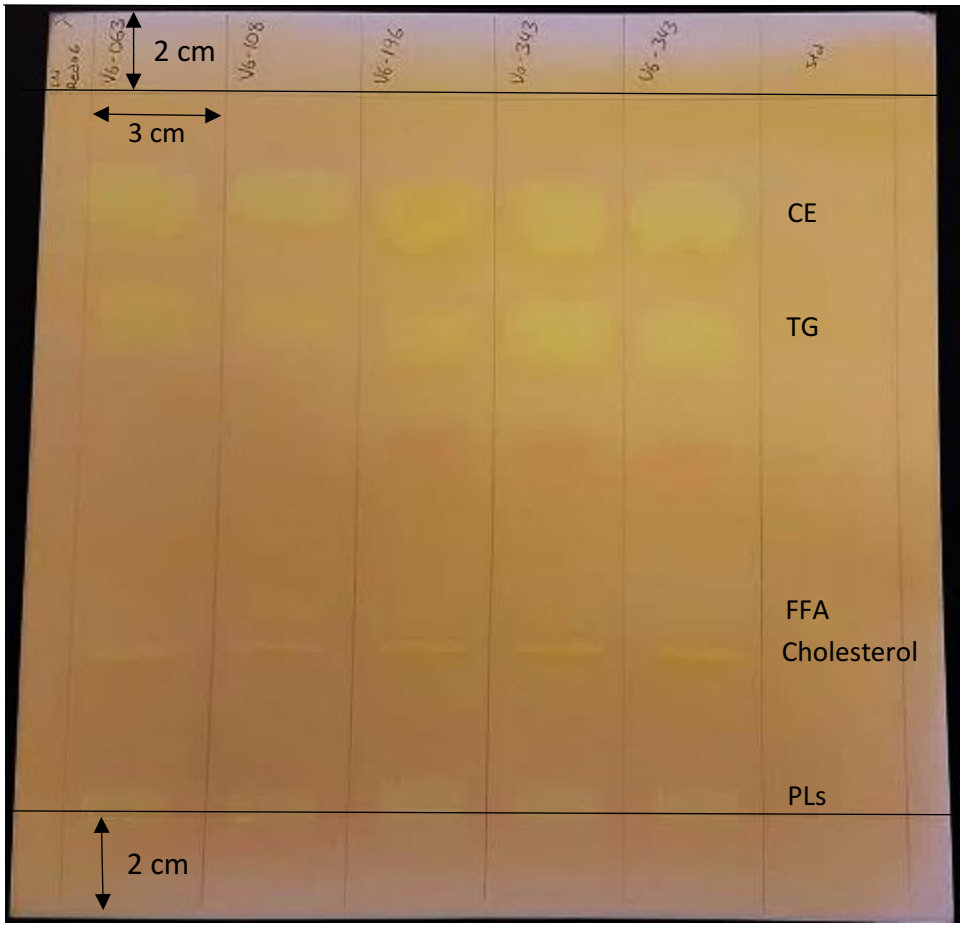

Figure 4 *First Thin Layer Chromatography. The samples were loaded onto the bottom drawn line, 2 cm from the bottom of the plate. The plate was taken out of the chamber when the solvent front migrated to the top drawn line which is 2 cm from the top of the plate. From top to bottom, the total lipids are separated into CE, TG, FFA, cholesterol and PLs.*

For the first TLC, a mixture of 170 mL of Petroleum ether, 2 mL of methanol, 5 mL of acetic acid and 30 mL ethyl ether was mixed into the TLC glass chamber (Cunnane et al., 2012). After mixing the solution, a 20 cm by 20 cm Whatman paper (3 MM Chr Chromatography Paper, GE healthcare Life Sciences, Fisher Scientific) was added to ensure the chamber maintains its solvent saturation. Around 40 minutes after the TLC plate was placed in the chamber, the solvent front reached the top line ensuring the samples had enough time for the lipids to separate into the different lipid compartments as illustrated above. When the solvent front reached the top line, the TLC plate was taken out of the chamber and remained in the fume hood to allow the solvent to evaporate from the plates. Afterward, the plate was sprayed using 0.02% 2,7-dichlorofluorescein

(Acros Organic, Fisher Scientific) in methanol to visualize the different lipid bands under ultraviolet light. Under the UV light, the different lipid bands were identified. Then the silica containing the lipid compartments was scraped and recovered into separate test tubes. The extraction of CE, TG and FFA from silica gel differed slightly from the extraction of PL from silica, therefore the procedure is separated in two different sections.

3.3.3 Extraction of CE, TG and FFA from silica gel

To extract CE, TG, and FFA from the silica gel, 2.5 mL of hexane, 2.5 mL of methanol and 5 mL of saturated NaCl in distilled water were added to the silica. The mixture was then vortex and centrifuged for six minutes at room temperature. The aqueous phase was separated from the organic, hexane phase. This allowed the lipids to be found in the top hexane layer and the silica to be at the bottom with the methanol and saturated NaCl water. The hexane layer was transferred to clean test tubes. CE required to be saponified (see section 3.3.5) before the methylation step whereas TG and FFA were methylated as seen in the flow chart below.

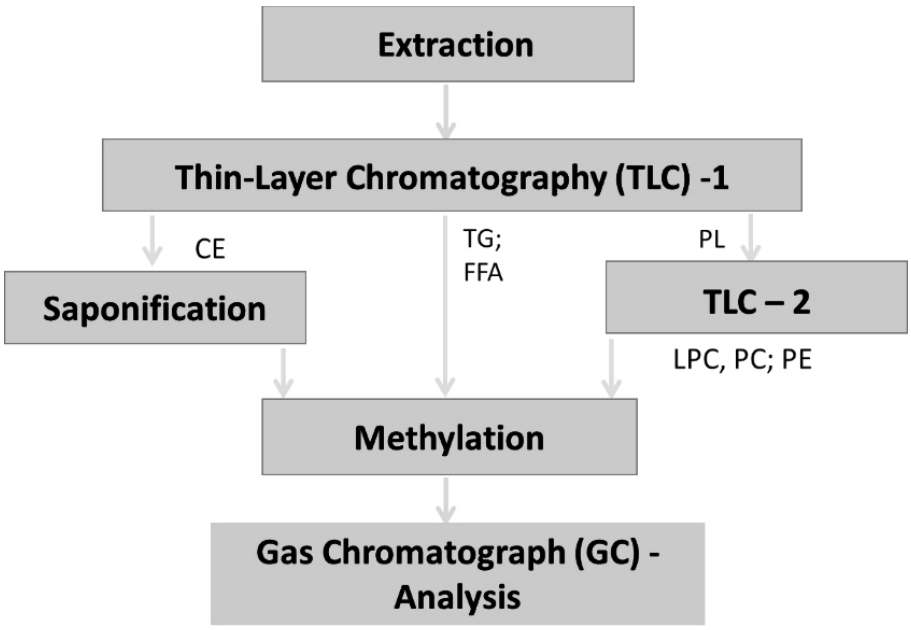

Figure 5 Flow chart of the different steps required to separate the lipids compartments and prepare the samples before fatty acid analysis by gas chromatography.

3.3.4 PL extraction from silica and TLC

Since a focus of this project is on PL compartments, total PL was separated using a second TLC plate. Before this, total PLs were extracted from the silica gel by washing the silica with 2.5 mL of chloroform, 2.5 mL of methanol and 0.5 mL of Millipore water. This procedure is different than the other lipid compartments because PLs are more polar than the other lipids. The test tubes containing the solvents and silica were vortexed for one minute and centrifuged for two minutes at 1800 rpm at room temperature. The supernatant was transferred to new test tubes. The silica left in the tubes was washed again with the same solvents and water, and supernatant from the second extract was combined in the new test tube. Thereafter, 5 mL of a solution of water with NaCl (0.9%) was added to the combined supernatant to allow the separation of the methanol and water from the chloroform phase. After vortexing the test tubes for one minute and centrifuging for 6 minutes at 1800 rpm at room temperature, the top methanol phase was removed, leaving the bottom chloroform phase with the PLs (Christie, 1982). The extracted PL were then dried under nitrogen stream before being transferred to a new thin layer silica plate using the same procedure as described above for further separation into LPC, PC and PE. Similar to the first TLC, the TLC plates were prepared prior to this step by heating the plate for 1 hour at 110 °C and then marking the lanes where the PLs were loaded. However, the composition of the migration solvent was this time composed of 100 mL of chloroform, 75 mL of methanol, 9 mL of acetic acid and 4 mL of Millipore water. Once the solvent was mixed well, a 20 cm by 20 cm Whatman paper was added to saturate the chamber evenly. The chamber was left to saturate for at least an hour before the TLC plate with the samples was placed in. The migration time for this separation lasted for about two hours. After migration, the plate dries under the fume hood for 10 minutes before being sprayed with 0.02% 2,7-dichlorofluorescein in methanol to be visualized under UV light. The PLs were separated into PE, PC, and LPC on the plate from top to bottom. Each PL compartment was extracted from the silica as described at the beginning of this section.

3.3.5 Saponification of CE

CE fractions were saponified to remove the cholesterol since it is not compatible with our chromatographic capillary column. Briefly, 3 mL of 5.6% potassium hydroxide in methanol, a strong base to hydrolyze the ester bond between the fatty acid chain and cholesterol was added to dried CE fractions. The tubes were then capped under nitrogen and heated for 30 minutes at 90 °C (Plourde, Tremblay-Mercier, Fortier, Pifferi, & Cunnane, 2009). The test tubes were then cooled down to room temperature for 15 min. Then, 5 mL of hexane and 2 mL of a solution of water with NaCl (0.9%) were added to the solution and centrifuged at 1800 rpm for 5 min at room temperature. The hexane layer on top containing the cholesterol was thereafter removed, leaving the fatty acid salts in the methanol/water phase in the tubes. To protonate the fatty acid salts, 300 uL of hydrochloric acid (36.5 – 38.0%, Fisher Chemical) was added making them neutral, free fatty acids. To solubilize the free fatty acids, 5 mL of hexane was added to the tubes and the mixture was then again centrifuged at 1800 rpm for 5 min at room temperature. The top hexane layer with the FFA was then transferred into clean tubes to be methylated.

3.3.6 Methylation of CE, TG, FFA, PE, PC, LPC

All separated lipid compartments were methylated to generate fatty acid methyl esters that are required for their analysis by gas chromatography. Prior to adding 3 mL of 14% boron trifluoride-methanol, the samples were dried under a nitrogen stream. The solution was then heated for 30 minutes at 90 °C as previously described (Plourde et al., 2009). After 30 min, tubes were cooled down to room temperature and then 5 mL of hexane and 2 mL of a solution of water with NaCl (0.9%) were added to solubilized fatty acid methyl esters. After centrifugation of the solution at 1800 rpm for 5 minutes at room temperature, fatty acid methyl esters in the hexane were collected and transferred into a new test tube. The hexane layer was then evaporated and a specific hexane volume for each lipid compartment was added: 330 µL hexane for CE, 380 µL hexane for TG, 120 µL of hexane for FFA, 85 µL of hexane for PE, 850 µL of hexane for PC and 50 µL of hexane for LPC. The differing volumes were required to make sure to reach an adequate limit of detection when performing gas chromatography.

3.3.7 Gas Chromatography

The fatty acid methyl esters from each lipid compartment were analyzed by gas chromatography as described in Plourde *et al.* (2009). Briefly, 1 uL of the sample was injected in splitless mode in the injector heated at 250 °C. The oven temperature containing the capillary column BPX-70 was maintained at 50 °C for 2 min and the oven temperature increased by 20 °C per minute to reach 170 °C and kept at this temperature for 15 minutes. Then, the temperature increased by 5 °C per minute to 210 °C and was maintained for 15 minutes. The stationary phase in this column was 70% cyanopropyl polysilphenylene siloxane and mobile phase was helium gas. The fatty acids affinity to the carrier gas differed between the hydrocarbon chain length. Shorter fatty acids would have a smaller retention time compared to longer fatty acids because shorter hydrocarbon chains have more affinity to the carrier gas and leave the column earlier. Therefore, different fatty acids will come out of the column at a different retention time. The flame ionization detector was set at 250 °C and detected the fatty acids coming out from the column by burning the compound with the flame. This provided a potential difference within the electrodes of the detector and this was recorded to generate peaks of the chromatogram. Greater amount of a fatty acid detected produced a greater area under the peak. Chromatograms were generated using the software OpenLAB CDS (ChemStation Edition for GC Systems, Agilent Technologies). Based on the retention time, the peak for each fatty acid were identified. The concentration of the fatty acids in each lipid compartment was calculated by comparing the area under its peak, to the area of a known precise concentration of internal standard that was added at the beginning of the experiment for that compartment. Therefore, by comparing the areas under the peak of fatty acids like DHA, EPA and ARA to the known internal standard concentration in each lipid compartment, the concentration of DHA, EPA and ARA in each lipid compartment for each sample can be calculated.

3.4 Statistical Analysis

This samples size of 25 APOE4 carriers and 25 non-carriers were selected based on the available APOE4 carriers we had in the cohort from the primary study. To ensure the sample size was sufficient, the sample size was calculated using the average delta (post – pre DHA diet) DHA concentration from the FFA and PL compartments from a study done by Chouinard-Watkin et al (Chouinard-Watkins et al., 2015). The delta DHA concentration was calculated by subtracting the DHA concentration of the high saturated fat with DHA diet by the high saturated fat only diet. The average delta DHA concentration in the FFA compartment for non-carriers was 1.95 ± 0.2 mg/L. The average delta DHA concentration in the FFA compartment for APOE4 carriers was 1.5 ± 0.1 mg/L. A sample size of 6 in each group will have 90% power to detect a difference in means of 0.450 (the difference between a Group of non-carriers delta DHA mean of 1.950 and a Group of carriers delta DHA mean of 1.500) assuming that the common standard deviation is 0.200 using a two group t-test with a 0.050 two-sided significance level. The same calculations were done to calculate the sample size using the total PL results from Chouinard-Watkins et al. (Chouinard-Watkins et al., 2015). The average delta DHA concentration in the PL compartment for non-carriers was 47.55 ± 4.7 mg/L. The average delta DHA concentration in the PL compartment for APOE4 carriers was 39.25 ± 3.8 mg/L. A sample size of 7 in each group will have 90% power to detect a difference in means of 8.300 (the difference between a Group of non-carriers delta DHA mean of 47.55 and a Group of carriers delta DHA mean of 39.25) assuming that the common standard deviation is 4.25 using a two group t-test with a 0.050 two-sided significance level. Therefore, we had enough statistical power with an n = 25 carriers and n = 25 non-carriers to limit making a type 1 error.

3.4.1 Baseline anthropometric measurements

The mean or median ± standard deviation of the baseline anthropometric measurements between non-carriers and carriers were compared to see if there was a difference. Firstly, a Shapiro-Wilk test was done to check if the variables were normally distributed. If the data was normally distributed, an un-paired, parametric student t-test was done to

compare the means. If the data was not normally distributed an un-paired, non-parametric Mann-Whitney test was done to compare the medians. The Mann-Whitney test was used for age, sex, BMI, glucose, total cholesterol, triglycerides, LDL-cholesterol, and vitamin B12 data. The student t-test was used for height, weight, and HDL-cholesterol data. Both the normality and mean or median comparison tests were analyzed using GraphPad Prism 9.

3.4.2 Primary analyses

To analyze statistical differences between DHA, EPA and ARA concentrations in the six lipid compartments a 2-way ANOVA or a non-parametric test was done using GraphPad Prism 9 depending on if the data was normally distributed. These data are expressed in mean ± standard error of the mean. The primary analyses compared the fatty acids concentrations based on genotype and diet. The dependent variable in this statistical model was the concentration of the fatty acid such as DHA, EPA or ARA in the respective lipid compartment (LPC, PC, PE, FFA, TG, and CE). The independent variables were the diet (pre- and post-supplementation) and APOE genotype (APOE4 non-carriers and carriers). The data used to check the normality was the delta (post – pre) concentration of DHA, EPA and ARA in the different lipid compartments. DHA and EPA in CE, EPA and ARA in PC, and ARA in PE were normally distributed and therefore a 2-way ANOVA was done to compare the means. The rest of the data was not normally distributed, and a non-parametric test was done to compare the medians. A Wilcoxon test, a non-parametric paired test was done to compare the pre- and post-supplementation concentrations by combining the data of APOE4 carriers and non-carriers. A Mann-Whitney test, non-parametric un-paired test was done to compare the fatty acid concentrations between APOE4 carriers and non-carriers by combining the pre and post data. A Mann-Whitney test (non-parametric, un-paired test) was done to analyse a genotype by diet interaction by comparing the delta (post-pre) fatty acid concentration between the APOE4 carriers and non-carriers.

3.4.3 Secondary analyses

A secondary analysis was done separating the data based on genotype and BMI. For these analyses the dependant variable was the delta concentration (post – pre) of the fatty acids. The independent variables were genotype (APOE4 non-carriers and carriers) and BMI (high and low BMI). Individuals who had a BMI lower than 25.2 kg/m^2 was put in the low BMI group and individuals with a BMI greater than 25.2 kg/m^2 was placed in the high BMI group. This cut-off value of 25.2 kg/m^2 was the median BMI of both APOE4 non-carriers and carriers. This value to separate the BMI groups is similar to what was used by Chouinard-Watkins et al. (25.5 kg/m^2) and Fisk et al. (25.2 kg/m^2) when they looked at the interaction of BMI to the plasma DHA concentration (Chouinard-Watkins et al., 2015; Fisk et al., 2018). Similar to the primary analysis a 2-way ANOVA was done to compare the means of DHA and EPA in CE, EPA and ARA in PC and ARA in PE based on genotype and in this case, BMI. For the other analyses a Mann-Whitney (non-parametric, un-paired) test was done. To analyze a BMI effect, the delta fatty acid concentration from the low and high BMI groups were compared by combining the data from APOE4 carriers and non-carriers. To analyze a genotype effect, APOE4 carriers and non-carriers delta fatty acid concentrations were compared by combining the BMI groups. All data are presented as mean ± SEM. P-value < 0.05 are considered statistically significant.

4. Results

4.1 Participant recruitment

In the main clinical study, 272 human participants aged between 18-80 years old living in Sherbrooke and its surroundings were recruited. Twenty-nine participants were excluded because they did not meet the inclusion criteria, refusal of participation or other reasons. Remaining 243 participants were randomized into two groups: placebo and omega-3 fatty acids. One hundred and twenty-two participants were randomized in the placebo group, but n = 26 participants discontinued, leaving 101 participants completing the study. In the omega-3 fatty acid group, 120 individuals were randomized in this group and n = 23 discontinued. Participants discontinued the study as they were not able to continuously take the supplements as required, due to hospitalization or personal reasons. Therefore, 97 participants completed the study in the omega-3 fatty acids group. In this add-on study, 25 APOE4 carriers under the omega-3 fatty acid supplement were matched to 25 non-carriers on the same supplement. Sixty-eight percent of the participants included in the add-on study are females.

4.2 Baseline anthropometric measurements based on genotype

Baseline characteristics of carriers and non carriers are presented in Table 4.1. There were no significant differences between the APOE4 carriers and non-carriers on the following anthropometric characteristics: sex, age, BMI, weight, height, glucose levels, TG levels, total cholesterol levels, HDL-cholesterol levels, LDL-cholesterol levels, and vitamin B12 levels (Table 4.1). However, a difference that could be clinically significant was seen in LDL-cholesterol levels where non-carriers had 2.94 ± 0.95 mmol/L and APOE4 carriers had 3.23 ± 0.79 mmol/L.

Table 4.1 Baseline anthropometric measurements of APOE4 carriers and non-carriers

	APOE4+	APOE4-	p-value
n	25	25	
% of females	68%	68%	> 0.9999
***Age (years)**	50 ± 17	51 ± 16	0.8285
***BMI (kg/m^2)**	25.9 ± 4.80	25.8 ± 4.01	0.9120
***Weight (kg)**	70.6 ± 14.08	70.5 ± 12.35	0.9814
***Height (cm)**	164.9 ± 7.52	165.3 ± 9.29	0.8810
***Glucose (mmol/L)**	4.40 ± 0.48	4.56 ± 0.57	0.3699
***TG (mmol/L)**	0.99 ± 0.54	0.96 ± 0.45	0.8739
***Total cholesterol (mmol/L)**	5.54 ± 1.04	5.02 ± 1.05	0.1871
***HDL – cholesterol (mmol/L)**	1.67 ± 0.45	1.64 ± 0.45	0.8483
***LDL – cholesterol (mmol/L)**	3.23 ± 0.79	2.94 ± 0.95	0.1110
***Vitamin B12 (pmol/L)**	357.5 ± 181.51	328.0 ± 121.51	0.8588

*Mean ± standard deviation

4.3 DHA, EPA and ARA concentrations in LPC, PC and PE

Figure 6 presents the levels of DHA, EPA and ARA in the LPC, PC and PE blood compartments. Plasma DHA, EPA and ARA concentrations were compared based on omega-3 fatty acid diet and APOE4 status. DHA and EPA concentrations in LPC and PC were 67-380% higher post-supplementation compared to pre-supplementation ($p < 0.0001$) and this was in both carriers and non-carriers of APOE4. The increase in DHA concentration in LPC post-supplementation from pre-supplementation seems to be greater in APOE4 carriers, but it was not significant potentially due to not having enough statistical power for this analysis. There was no supplement effect on the level of ARA in

the plasma LPC compartment, but there was a genotype trend ($p = 0.0842$) where APOE4 carriers had higher levels of ARA than non-carriers. In the PC lipid compartment, there was a diet effect for ARA concentration where ARA levels were 25% lower in both carriers and non -carriers ($p = 0.0002$). There was also a diet effect for DHA and EPA concentrations in PE with $p = 0.0002$ and $p < 0.0001$, respectively. There was a trend in the genotype by diet interaction for ARA concentration in PE ($p = 0.0866$), where APOE4 carriers post-supplementation with omega-3 fatty acids had a greater decrease in ARA levels compared to non-carriers.

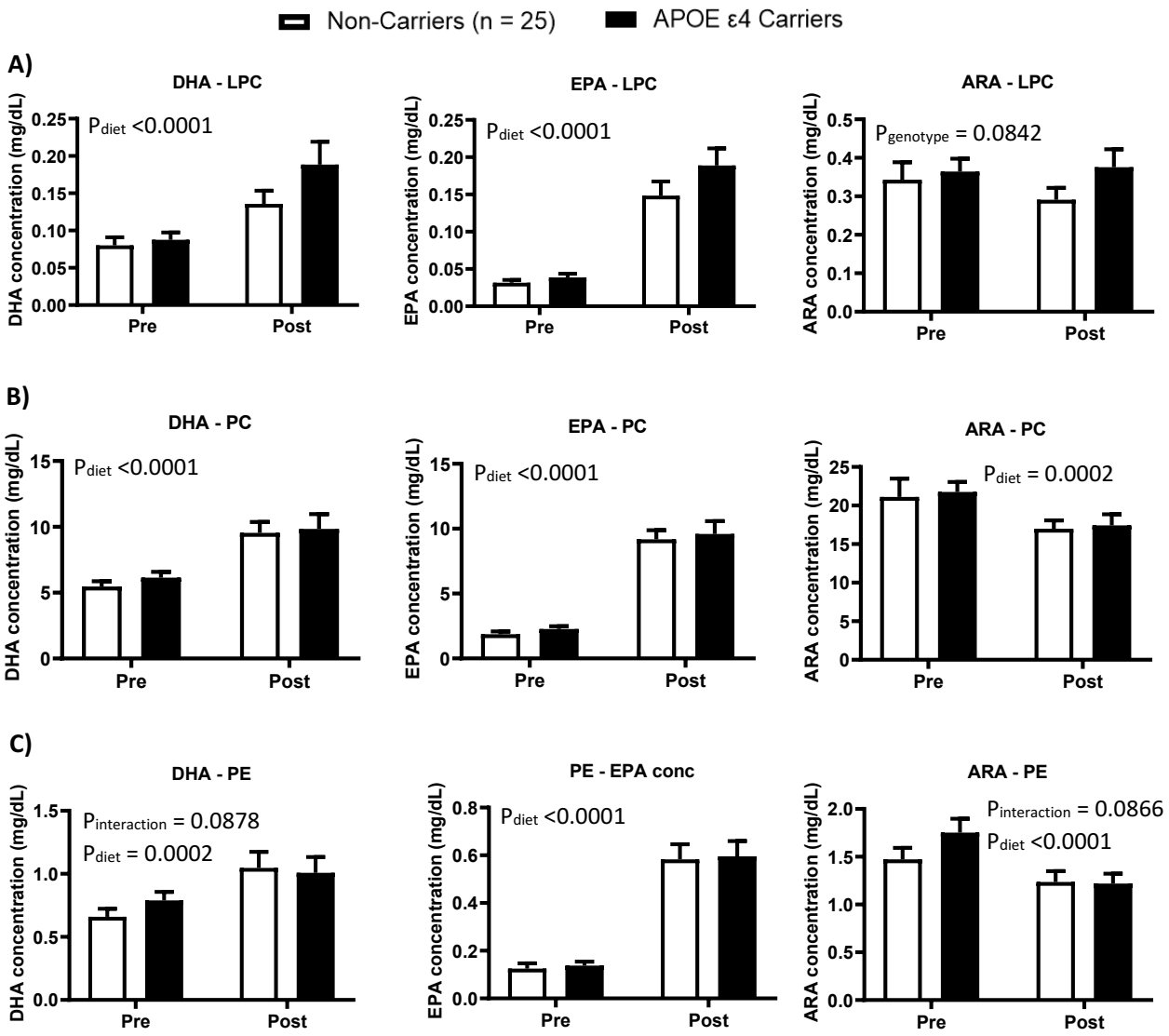

Figure 6 Mean (±SEM) plasma DHA, EPA and ARA concentrations in LPC, PC and PE. Plasma samples pre and post omega-3 fatty acid supplementation for six months in APOE4 carriers and non-carrier were analyzed. In all the graphs, non-carriers' data are shown in white and APOE4 carriers' data are shown in black. On the left side of all the graphs, the fatty acid concentration pre-supplementation is shown and on the right is post-supplementation concentrations. The concentration of the three fatty acids are shown in mg/dL. A) LPC (n=25 APOE4 carriers; n=25 non-carriers): DHA and EPA had a significant diet effect of p < 0.0001 for both. ARA had a genotype trend of p = 0.0842. B) PC (n=25 carriers; n=24 non-carriers): DHA and EPA had significant diet effect where p <0.0001 for both. ARA had a significant diet effect with p = 0.0002. C) PE (n=25 APOE4 carriers; n=25 non-carriers): DHA had diet effect with p = 0.0002. EPA had a diet effect with p < 0.0001. ARA had a diet effect with p <0.0001 and a genotype by diet trend (p = 0.0866).

4.4 DHA, EPA and ARA concentrations in FFA, TG and CE

Figure 7 presents the levels of DHA, EPA and ARA in the FFA, TG and CE blood compartments. In the FFA lipid compartment, DHA and EPA were 67% and 260% higher

post-supplementation compared to baseline ($p = 0.0002$ and $p < 00001$ respectively). There was no genotype, no diet nor interactions for ARA concentration in FFA class. In the TG lipid compartment, there was a diet effect for DHA and EPA ($p < 0.0001$ for both) and again there was no significant difference for ARA in TG. There was a genotype trend for DHA concentration in CE ($p = 0.0883$) and there was a diet effect for DHA and EPA concentrations in CE ($p < 0.0001$ for both). There was a genotype effect for ARA concentrations in CE ($p = 0.0434$).

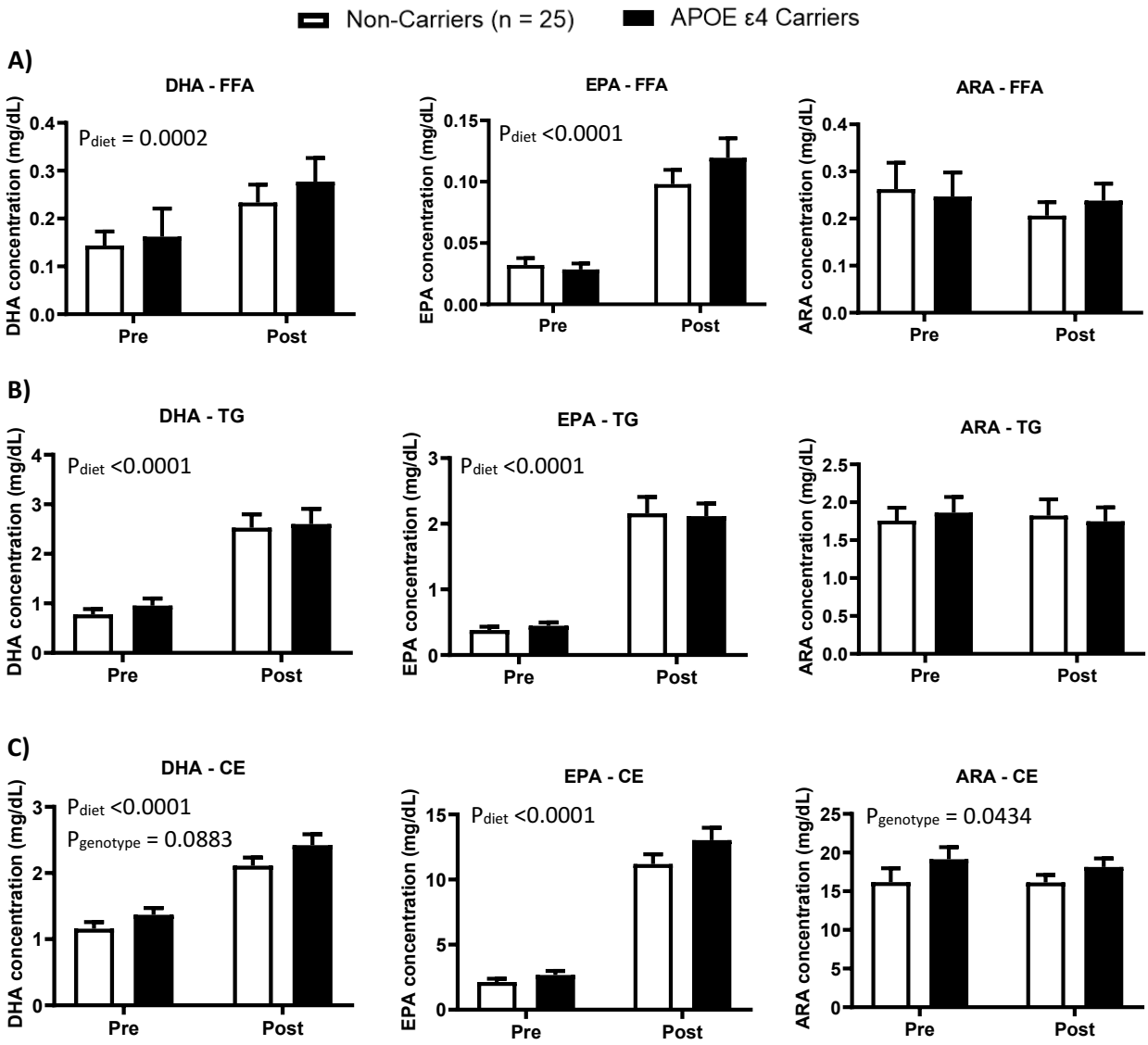

Figure 7 Mean (±SEM) plasma concentrations of DHA, EPA and ARA in FFA, TG and CE before and after omega-3 fatty acid supplementation for six months. Data for pre-supplementation is shown on the left and post-supplementation is shown on the right. Non-carriers' data are shown in white and APOE4 carriers' are shown in black. A) FFA (n=25 APOE4 carriers; n=25 non-carriers): DHA and EPA concentrations had a diet effect of p = 0.0002 and p < 0.0001, respectively. B) TG (n=25 APOE4 carriers; n=25 non-carriers): DHA and EPA had a diet effect of p <0.0001. C) CE (n=25 APOE4 carriers; n=25 non-carriers): DHA and EPA had a diet effect of p <0.0001. A genotype trend with p = 0.0883 was present for DHA concentration in CE. For ARA in CE had a genotype effect (p= 0.0434).

4.5 Secondary analysis based on BMI and genotype

Looking at the omega-3 fatty acids, DHA and EPA in LPC results shown in Figure 6, APOE4 carriers seem to have higher plasma omega-3 fatty acid levels than non-carriers post-supplementation even though it is not significant. To explain this observation, a secondary analysis was done splitting APOE4 carriers and non-carriers into low and high BMI group.

The rationale for this choice is motivated by a previous study done by our group supporting a genotype by BMI interaction for DHA in phospholipids and total lipids, and for EPA in neutral and total lipids (Chouinard-Watkins et al., 2015).

For the secondary analysis, individuals who had a BMI lower than the median BMI of APOE4 carriers and non-carriers, 25.2 kg/m^2, were allocated to the low BMI group. Individuals who had a BMI greater than 25.2 kg/m^2 were allocated to the high BMI group. In non-carriers, there was n = 11 in the low BMI group and n = 14 in the high BMI group. In APOE4 carriers, there was n= 14 in the low BMI group and n= 11 in the high BMI group. The ΔDHA, ΔEPA and ΔARA concentrations were compared between the genotype and BMI groups. Δ concentration is the post concentration minus the pre concentration of the fatty acid being analyzed in their lipid compartment.

4.5.1 Baseline anthropometric measurements based on BMI

Firstly, baseline characteristics of individuals in the low and high BMI groups are presented in Table 4.2. The anthropometric characteristics were not split based on genotype in this table as no differences were seen between the genotypes in the table 4.1, and in the DHA and EPA levels in the different lipid compartments in figure 6 and 7. As shown in table 4.2 there were statistical differences between the low and high BMI groups on the following anthropometric characteristic characteristics: BMI, weight, glucose levels, TG levels, HDL-cholesterol levels, and vitamin B12 levels. The high BMI group had a greater BMI, weight, glucose levels and TG levels, and had lower HDL-cholesterol and vitamin B12 levels compared to the low BMI group. There were no statistical differences between the low and high BMI groups on the following anthropometric characteristics: genotype, sex, age, height, total cholesterol levels, LDL-cholesterol levels, DHA in TG levels and DHA in FFA levels (Table 4.2). A trend for the difference in age between the BMI groups was seen (p = 0.0558). There are other differences that are not statistically demonstrated in the amount of APOE4 carriers to non-carriers, number of females, LDL-cholesterol levels present in each BMI group.

Table 4.2 Baseline anthropometric measurements of low and high BMI groups

	Low BMI	High BMI	p-value
n	25	25	
Genotype (APOE4 : non-carriers)	14:11	11:14	0.5721
% of females	72%	64%	0.7624
***Age (years)**	47 ± 16	54 ± 16	0.0558
***BMI (kg/m^2)**	22.3 ± 1.65	29.5 ± 3.10	<0.0001
***Weight (kg)**	61.0 ± 7.87	80.0 ± 10.09	<0.0001
***Height (cm)**	165.3 ± 7.64	164.8 ± 9.19	0.8365
***Glucose (mmol/L)**	4.28 ± 0.44	4.68 ± 0.54	0.0043*
***TG (mmol/L)**	0.78 ± 0.36	1.18 ± 0.56	0.0007*
***Total cholesterol (mmol/L)**	5.03 ± 0.82	5.34 ± 1.23	0.4973
***HDL – cholesterol (mmol/L)**	1.82 ± 0.39	1.49 ± 0.43	0.0065*
***LDL – cholesterol (mmol/L)**	2.85 ± 0.61	3.31± 1.04	0.1178
***Vitamin B12 (pmol/L)**	363.2 ± 180.43	322.3 ± 121.42	0.0226*

***Mean ± standard deviation

4.5.2 Δ concentration of DHA, EPA and ARA in LPC, PC and PE based of genotype and BMI

Figure 8 presents the ΔDHA, ΔEPA and ΔARA levels in the LPC, PC and PE blood compartments. When comparing the ΔDHA concentration in LPC between the low and high BMI, there was a significant difference (p = 0.0211). The ΔDHA concentration was 168% higher in the low BMI group compared to high BMI group. For the ΔEPA concentration in LPC, there was a BMI effect (p = 0.0025) where low BMI group had 79% greater increase post-supplementation compared to high BMI group. There was no

genotype nor BMI effect for ΔARA concentration in LPC. In PC, there was no significant changes whatsoever for ΔDHA and ΔARA levels. There was a BMI trend for ΔEPA in PC ($p = 0.0611$) For PE, no significant changes were seen for ΔEPA and ΔARA besides a genotype trend for ΔDHA concentration ($p = 0.0878$).

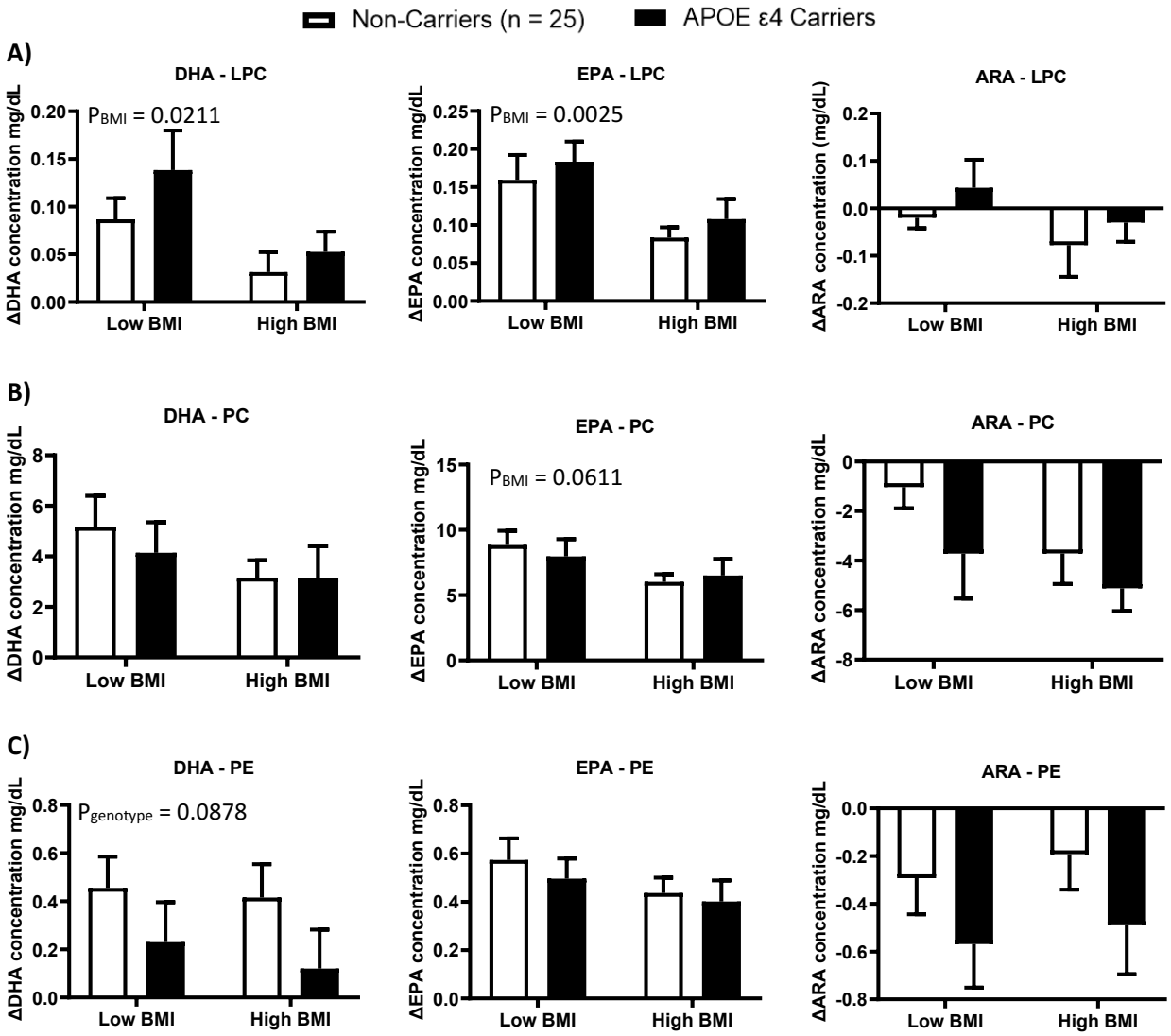

Figure 8 ΔMean (±SEM) DHA, EPA and ARA plasma concentrations in LPC, PC and PE. Δ concentration on the y-axis is the post-supplementation concentration minus the pre-supplementation concentration. Data are separated based on genotype and BMI. Non-carriers are shown in white and APOE4 carriers are shown in black. Data from the low BMI group (< 25.2 kg/m^2) are shown on the left and data from the high BMI group (<25.2 kg/m^2) are shown on the right for all graphs. A) LPC (Low BMI (n = 11 non-carriers; n = 14 APOE4 carriers), High BMI n = 14 non-carriers, n = 11 APOE4 carriers)): BMI effect was observed in ΔDHA and ΔEPA with p = 0.0211 and p = 0.0025, respectively. B) PC (Low BMI (n = 11 non-carriers; n = 14 APOE4 carriers), High BMI n = 13 non-carriers, n = 11 APOE4 carriers)): There is a BMI trend for ΔEPA in PC with p = 0.611. No difference was seen for ΔDHA and ΔARA levels. C) PE (Low BMI (n = 11 non-carriers; n = 14 APOE4 carriers), High BMI n = 14 non-carriers, n = 11 APOE4 carriers)): No significant BMI nor genotype effect was seen for ΔDHA, ΔEPA and ΔARA. There was a genotype trend with p = 0.0878 for ΔDHA.

4.5.3 Δ concentration of DHA, EPA and ARA in FFA, TG and CE based of genotype and BMI

Figure 9 presents the ΔDHA, ΔEPA and ΔARA levels in the FFA, TG and CE blood compartments. There were no significant differences in ΔDHA, ΔEPA and ΔARA

concentrations based on genotype and BMI in the FFA, and TG lipid compartments. In the CE lipid compartment, there was a genotype by BMI interaction trend for ΔDHA (p = 0.0585). There was a BMI effect for ΔEPA in CE (p = 0.0456), where low BMI group had 26% greater increase post-supplementation compared to the high BMI group. There was no significant difference in ΔARA concentration in CE.

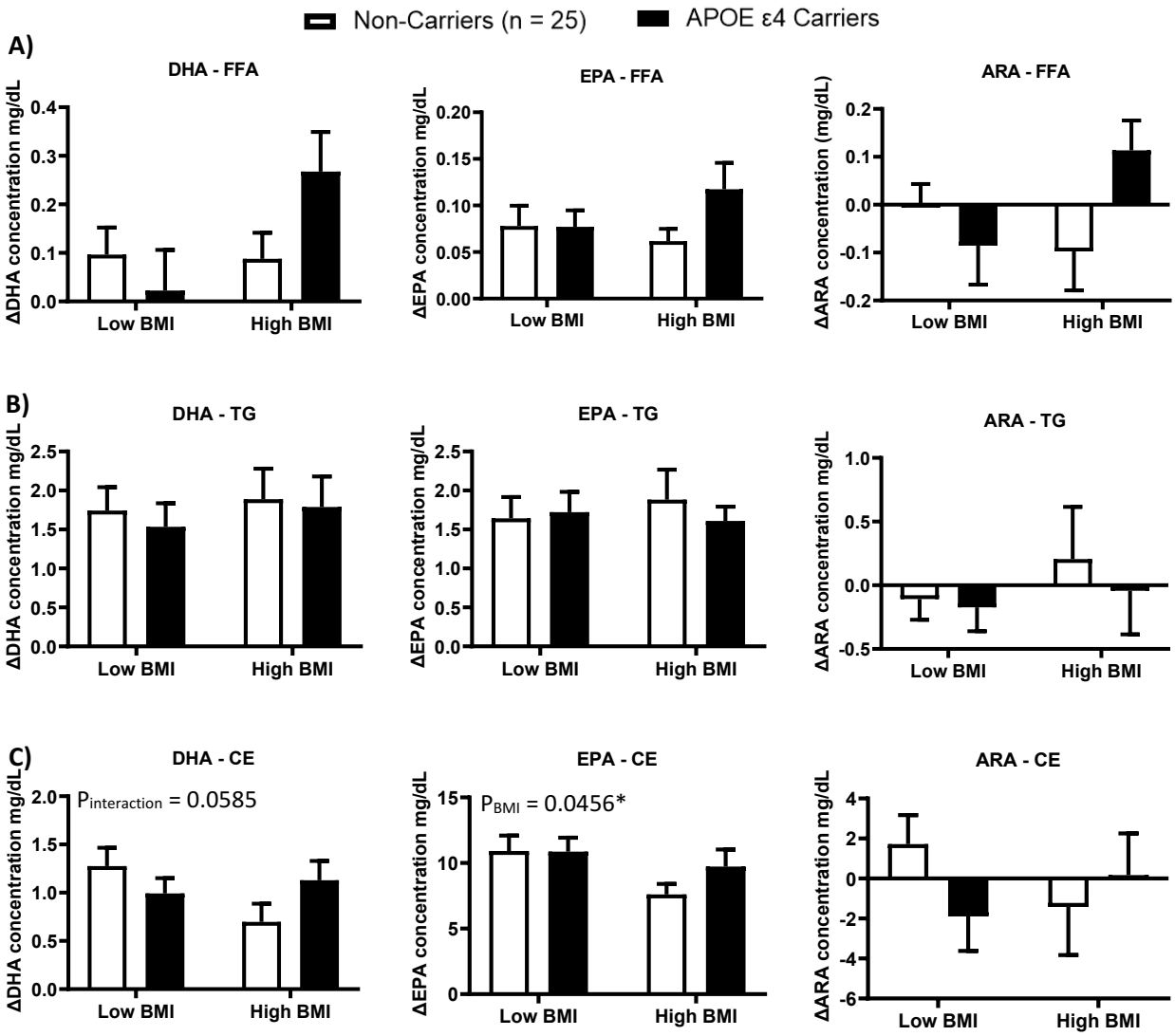

Figure 9 ΔMean (±SEM) DHA, EPA and ARA concentration in FFA, TG and CE. Data of non-carriers are shown in white and APOE4 carriers are shown in black. Data for non-carriers and carriers are separated based on BMI: Low BMI group < 25.2 kg/m² (n = 11 non-carriers; n = 14 APOE4 carriers); High BMI group > 25.2 kg/m² (n = 14 non-carriers; n = 11 APOE4 carriers). A) FFA: No significant difference in ΔDHA, ΔEPA and ΔARA based on genotype and BMI. B) TG: No significant effect present in ΔDHA, ΔEPA and ΔARA in the TG lipid compartment. C) CE: There was a BMI by genotype interaction trend for ΔDHA with p = 0.0585. For ΔEPA there is a BMI effect with p = 0.0456. For ΔARA in CE there is no difference between the genotype and BMI groups.

5. Discussion

For this add-on study, we hypothesized there is a compartment packaging issue of DHA in APOE4 carriers. More precisely, after omega-3 fatty acid supplementation for six months, the concentration of DHA and EPA will not increase in APOE4 carriers compared to non-carriers in FFA and LPC, the lipid compartments the brain can take-in fatty acids. Additionally, DHA is packaged more in the TG compartment in APOE4 carriers compared to non-carriers. In our study, we found after consuming 3.2 g of omega-3 fatty acids (1.4 g of DHA and 1.8 g of EPA) everyday for six months, plasma levels of DHA and EPA increased in both APOE4 carriers and non-carriers in the six plasma lipid compartments analysed. There was a genotype by diet trend for DHA and ARA concentrations in PE. Pre-supplementation non-carriers had less DHA in the plasma PE compartment compared to carriers of APOE4, however after omega-3 fatty acid supplementation, non-carriers had greater DHA levels in plasma PE compared to non-carriers. Therefore, post-supplementation non-carriers had a greater increase in DHA levels in PE compared to APOE4 carriers. However, APOE4 carriers had greater levels of DHA in the CE compartment in both pre- and post-supplementation compared to non-carriers. Based on these results, our first hypothesis that there is a compartment packaging issue of DHA and EPA in the plasma in APOE4 carriers is partly true. There is a compartment packaging issue of DHA, but not of EPA. The second hypothesis that after supplementation with omega-3 fatty acids for six months, the concentration of DHA and EPA will not rise in APOE4 carriers compared to non-carriers in the lipid compartments the brain can take-in fatty acids (FFA and LPC) is false because we saw an increase in all compartments. In the next few subsections, potential explanations for the results will be provided.

5.1 DHA and EPA concentrations increase with omega-3 fatty acid supplementation

DHA is important for normal brain function as it can be oxidized into metabolites involved in neuroprotection and anti-inflammation (Lacombe et al., 2018). It is also involved in synaptic plasticity where it is concentrated in the synapses, maintaining the membranes of neurons, producing and clearing amyloid-β, and vascular health (Yassine, et al., 2017). Higher plasma omega-3 fatty acid concentrations, such as DHA and EPA are associated

with better cognitive functions (Boudrault et al., 2009; van der Lee et al., 2018). Our brain is incapable of forming DHA and therefore relies on the peripheral circulation (Plourde & Cunnane, 2007). Studies done in rats found the brain can take-in DHA in the LPC and FFA form (Chen et al., 2015). Based on the current study results, omega-3 fatty acids, DHA and EPA plasma concentrations in the six lipid compartments (LPC, PC, PE, FFA, TG, and CE) increased post-supplementation compared to pre-supplementation. Both the APOE4 carriers and non-carriers benefitted from the taking the omega-3 fatty acid supplements for six months because more DHA was available in their plasma, hence more DHA was available to reach the brain (as shown in figure 10). Therefore, taking omega-3 fatty acid supplementation might contribute to maintaining cognitive ability or prevent its decline over time as previous epidemiology studies have reported (Boudrault et al., 2009; Freund-Levi et al., 2006; Pontifex et al., 2018). A study by Donadio et al. gave a similar dosage of omega-3 fatty acids as our study, 1.47 g of DHA and 1.88 g of EPA to thirty participants (Donadio, et al., 2006). They looked at DHA, EPA and ARA percent to total fatty acids in the PL compartment at baseline and six months. They had higher DHA, EPA and ARA% to total fatty acids in the PL compartment after six months of omega-3 fatty acid supplementation (Donadio et al., 2006). Similar results were seen in a study by Fisk et al. where adults had consumed oily fish, which is a good source of DHA and EPA for eight weeks. They found after consuming oily fish, the concentration of DHA increased in all the lipid compartments they looked at, PC, NEFA, CE and TG. The concentration of EPA increased in PC and TG with a trend in CE (Fisk et al., 2018). To our knowledge, this current study is the first to look at plasma DHA, EPA and ARA concentrations in the LPC and PE lipid compartments before and after omega-3 fatty acid supplementation.

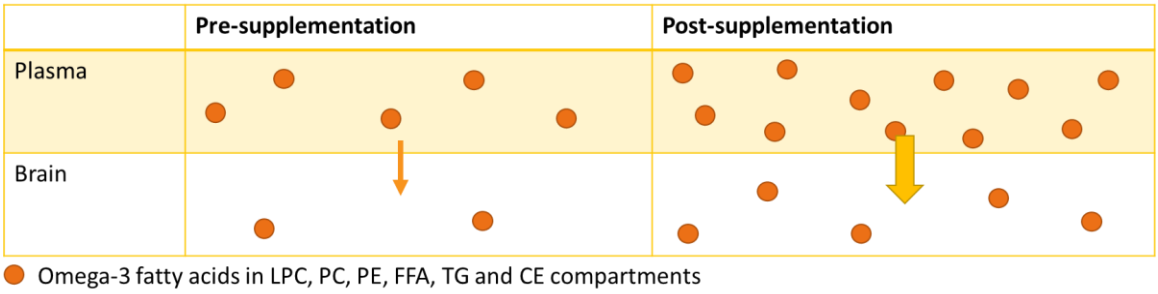

Figure 10 Increased omega-3 fatty acid levels in the FFA and LPC form in the plasma post-supplementation can mean more DHA and EPA would get transported to the brain when the brain needs to replenish the DHA loss during nerve damage. Both DHA and EPA in the LPC, PC, PE, FFA,TG and CE compartment are represented in the figure as orange circles.

5.2 APOE4 carriers have altered DHA and ARA levels in CE

Carrying the APOE4 allele increases the risk of developing Alzheimer's disease (Hauser et al., 2011). A review by Davignon, stated APOE4 allele is associated with higher total cholesterol and LDL cholesterol (Davignon, et al., 1988). Even though significant differences were not seen in our results in the total cholesterol and LDL-cholesterol levels in APOE4 carriers compared to non-carriers (p= 0.1817), the total cholesterol and LDL-cholesterol levels were higher in APOE4 carriers. These differences were not for sure as statistical differences were not demonstrated but, the statistical power was probably not sufficient for these comparisons. There was a genotype difference of DHA and ARA in the CE compartment where APOE4 carriers had higher levels. CE is found in the core of lipoproteins with TG and hydrophobic vitamins (Iqbal & Hussain, 2009). In intestinal cells, chylomicrons are formed and through exocytosis they enter the lymphatic vessels before it goes into the blood circulation. In the lymphatic vessels, around seventy-five percent of cholesterol is in the esterified form (Iqbal & Hussain, 2009). This suggests the esterification of cholesterol was important for when a lot of cholesterol was being packaged into chylomicrons (Iqbal & Hussain, 2009). Perhaps a similar principle is applied in the plasma to explain the higher levels of DHA and ARA in the plasma CE seen in our results. To prevent more cholesterol from entering cells following a diffusion gradient, potential excess cholesterol is esterified to fatty acids like DHA and ARA.

5.3 ARA concentration altered in PC and PE

ARA is a pro-inflammatory metabolite. Plasma ARA concentrations did not change in the LPC, FFA, TG and CE lipid compartments post-supplementation. However, the ARA concentration in PC and PE decreased post-supplementation. This can mean with greater levels of DHA and EPA in the plasma, more ARA in PC and PE are being oxidized or stored in the membrane of cells. Since a diet effect is not seen in the other lipid compartments for ARA, it can be assumed ARA from PC and PE are not being remodelled more into LPC, FFA, TG and CE than usual post-supplementation. Additionally, for ARA concentration in PE, there was a genotype by diet trend. APOE4 carriers before supplementation had higher ARA levels than non-carriers. After omega-3 fatty acid supplementation ARA levels decreased in both APOE4 carriers and non-carriers, and ARA levels were similar. Prior to taking omega-3 fatty acid supplementation APOE4 carriers were more susceptible for pro-inflammatory mechanisms as APOE4 carriers had higher ARA levels. This was corrected after omega-3 supplementation, where ARA levels in APOE4 carriers became similar to that of non-carriers in PE.

5.4 Does BMI alter the uptake of DHA and EPA in plasma?

Having a high BMI is a risk factor for cognitive impairment. When comparing the ΔDHA concentration (post – pre concentration) between the low and high BMI groups, a BMI effect was seen in LPC and a BMI by genotype trend in CE. When comparing the ΔEPA concentration (post – pre concentration) between the low and high BMI groups, there was a BMI effect in LPC and CE and a BMI trend in PC. Since the y-axis represents the post minus the pre concentration of the fatty acid, greater Δ concentration means, greater the fatty acid levels increased in the plasma post-supplementation. Therefore, individuals in the high BMI group had a smaller increase of EPA in LPC, PC and CE and DHA in LPC after taking the omega-3 fatty acid supplements whereas individuals in the low BMI group had a greater increase. Figure 11 will help visualize these results. Results we obtained were similar to what Chouinard Watkins et al. obtained where they compared the ΔDHA ΔEPA and ΔARA concentration between a low and high BMI group carrying or not the APOE4 genotype in FFA, phospholipids, neutral lipids (TG+CE) and total lipids (Chouinard-Watkins

et al., 2015). They defined the low BMI group being under 25.5 kg/m^2 and high BMI group having a BMI greater than 25.5 kg/m^2. They reported a genotype by BMI interaction for ΔDHA and ΔEPA in phospholipids, neutral lipids, and total lipids. The results from Chouinard-Watkins were not looking at the same lipid compartments as the current study except for FFA. We both did not find a BMI nor genotype effect for FFA. In our study, we saw a genotype by diet trend for DHA in CE, and a BMI effect for DHA in LPC and EPA in LPC, PC and CE. In the other lipid compartments, we did not see a BMI effect. The differences in our results could be explained by the different diets given to the participants. They had given a high saturated fat diet with the omega-3 fatty acid diet to their participants, where we only gave omega-3 fatty acid supplements. Another study found no differences in DHA and EPA concentrations after consuming 0.1 to more than 2 portions of oily fish such "as salmon, herring, mackerel, fresh tuna, sardines, kippers and trout" for eight weeks between the BMI groups in the lipid compartments PC, FFA, CE and TG (Fisk et al., 2018). This is not consistent with our results seen for ΔEPA in CE but, one potential explanation resides in the way they compared their results since they only compared the post concentrations between groups instead of the delta of post and pre supplementation like we did. Hence, they did not account for baseline concentrations in their analyses. Moreover, there was a difference in the diet their participants consumed to ours. Their diet was based on fish intake and our study was based on omega-3 fatty acid supplements. Moreover, the time length participants had to consume an omega-3 rich diet in their study lasted for eight weeks and ours was six months. To our knowledge, we are the only group that have investigated the interaction between BMI and genotype on ΔDHA, ΔEPA and ΔARA concentrations in the LPC, and PE lipid compartments.

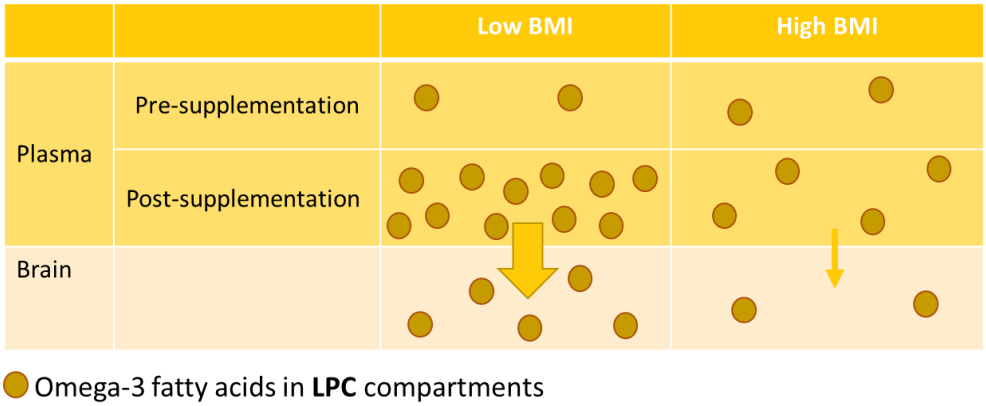

Figure 11 Omega-3 fatty acid levels in LPC pre and post-supplementation. The brown circles represent the omega-3 fatty acids in the LPC compartment. To better understand the results, in this figure the omega-3 concentration pre-supplementation is the same for individuals in the low and high BMI groups. Post-supplementation individuals from the low BMI group have a greater increase of omega-3 fatty acid concentration compared to individuals in the high BMI group. Greater increase of omega-3 fatty acids in the low BMI group means more omega-3 would be able to enter the brain. Since in the high BMI group there was a lesser increase of omega-3 levels, less omega-3 would be available to enter the brain compared to the low BMI group.

A possible explanation to why the high BMI group had less of an increase in DHA and EPA in certain plasma lipid compartments can be the total blood volume in these individuals were higher than the low BMI group, and therefore the distribution of DHA and EPA is diluted. A study by Cepeda-Lopez measured the total blood volume of normal weight (n = 24; BMI = 18.5 to 24.9 kg/m^2), overweight (n = 20; BMI = 25 to 29.9 kg/m^2) and obese (n = 20; BMI = 30 to 39.9 kg/m^2) females. They found the total blood volume was greater in overweight and obese individuals compared to normal weight females. They also found serum iron mass was lower in overweight and obese individuals compared to normal weight individuals (Cepeda-Lopez et al., 2019), similar to how we saw a lesser increase in DHA and EPA concentration in those with a high BMI. Additionally, DHA and EPA are lipophilic molecules. A common hypothesis to explain the lower increase in lipophilic molecules like vitamin D in obese individuals, is that it is diluted in the fat, serum, liver, and muscle volumes (Vranić, et al., 2019; Zakharova et al., 2019). Therefore, a potential reason to why DHA and EPA levels did not increase as much in the high BMI group is that it is diluted within the total fat and serum volumes.

High BMI is inversely associated to insulin sensitivity (Walton et al., 1992). In obese individuals, adipocytes that store TG become big in size and TG starts to be stored around other organs that normally would have small amounts of fat (Snel et al., 2012). This excess lipid collection in non-adipose tissue is due to lipotoxicity effect (Schaffer, 2003). Non-adipose tissues can store a limited amount of lipids. When this threshold is surpassed lipotoxic consequences can happen such as cellular dysfunction and cell death (Schaffer, 2003). Lipotoxic effects in the beta cells of the pancreas can cause dysregulation in the secretion of insulin (Rasouli, et al., 2007). Build-up of excess FFA can additionally cause apoptosis of the beta-cells (Schaffer, 2003). In our study, even though we excluded individuals diagnosed with diabetes, individuals in the high BMI group had higher glucose and total TG levels than individuals in the low BMI group suggesting individuals in the high BMI group might be in early stages of lipotoxicity. However, ΔDHA and ΔEPA in TG were not different between the low and high BMI groups, suggesting the total TG difference seen can be caused by other fatty acids.

5.5 Strength

A strength of this project is that it is a long-term project as the participants took omega-3 fatty acid supplements for six months. This study was also a double-blind study meaning the participants did not know whether they were consuming the placebo or omega-3 fatty acid supplements and neither did the research team providing the supplements to participants. Strengths more specifically to the add-on study is that the DHA, EPA and ARA concentrations were analyzed in the different lipid compartments of the plasma. Instead of analyzing these fatty acids in the total lipids or neutral lipids (that encompasses CE and TG) or total PLs, we separated these categories and looked at LPC, PC, PE, FFA, TG and CE separately. Furthermore, we demonstrated DHA, EPA and ARA concentrations individually, without having them on the y-axis as ratios like EPA:DHA or DHA:ARA or EPA:ARA. This way it can be seen if the fatty acid is increasing or decreasing without assuming the other fatty acid is constant or increasing or decreasing. In some studies they show an increase in DHA:ARA levels, but we would not know if it is due to an increase in DHA levels and decrease in ARA levels, or increase in DHA and ARA levels is constant, or if

DHA levels is constant and ARA levels decreased. We analyzed the fatty acid concentration based on the omega-3 fatty acid supplementation and based on genotype. Additionally, we analysed the differences in the delta (post – pre) fatty acid concentration based on BMI and genotype.

5.6 Limitation

Even though in this study we looked at pre and at six months mark of taking omega-3 supplements, plasma samples were collected every month as the participant were taking the supplements. A limitation was not having enough time to analyze the plasma samples at the different time points to evaluate the pharmacodynamic of the omega-3 fatty acid supplements. Another limitation to this study is that no follow-up was done a year or few years after the end of the study to see whether any participants developed mild cognitive impairment, dementia, or Alzheimer's disease. With that information, we could have done further analyses to see if lower DHA or EPA in PL compartments could be a potential biomarker for mild cognitive impairment or Alzheimer's disease as suggested by Abdullah and colleagues (Abdullah et al., 2017). Moreover, we could have compared the results to LPC levels as Mapstone and colleagues suggested low plasma LPC can predict mild dementia and Alzheimer's disease with ninety percent precision within 3 years (Mapstone et al., 2014). An additional limitation was using TLC to separate the total lipids into the different lipid compartments as there is more room for variations as it is a long process compared to using liquid chromatography (LC). An additional limitation was not having a sufficient sample size to analyze the results based on sex. Furthermore, as it was mentioned in the results and discussion section, some differences between groups were seen, but they were not statistically significant. There was probably insufficient statistical power for these comparisons. The power of our study mentioned in the statistical analyses section was determined using other results and not the actual results presented, and it was not determined for all the analyses presented in this thesis. Another limitation is not being able to do fatty acid profiles of certain brain regions to see if the higher omega-3 fatty acid levels seen in the plasma correlates to how much DHA is taken up by the brain. It also would have been interesting to quantify the amount of Mfsd2a

transporters present on the epithelial brain membrane of the blood brain barrier and if the amount varied between APOE4 carriers and non-carriers.

5.7 Conclusion

In this add-on study, we showed after omega-3 fatty acid supplementation for six months DHA and EPA concentrations increased in both APOE4 carriers and non-carriers in the following lipid compartments: LPC, PC, PE, FFA, TG, and CE. APOE4 carriers had higher ARA concentration and a trend of higher DHA concentration in the plasma pre- and post-supplementation compared to non-carriers. Additionally, when we evaluated the Δconcentrations based on APOE4 status and BMI, non-carriers in the high BMI group had a lower ΔDHA trend in the CE compartment compared to non-carriers in the high BMI group and compared to APOE4 carriers from both BMI groups. ΔEPA in CE was lower in those with a high BMI compared to low BMI in both APOE4 carriers and non-carriers. ΔDHA and ΔEPA concentrations in LPC were lower in individuals with a high BMI compared to individuals with a low BMI. LPC is one of the compartments the brain can take-in fatty acids. In individuals with a high BMI, there was a little increase in DHA and EPA levels after consuming omega-3 fatty acids compared to individuals in the low BMI. Therefore, if less plasma DHA is available in one of the compartments the brain can obtain, less DHA would be available to replenish the DHA used by the brain. Individuals with a high BMI might potentially need a higher dose of omega-3 fatty acid to have a greater increase in the plasma DHA and EPA concentrations.

5.8 Future Perspective

The current add-on study found individuals with a high BMI had lower levels of DHA and EPA in the LPC compartments. This can suggest less DHA is available for the brain uptake as the brain incorporate DHA in the non-esterified and LPC compartments. In an animal study, more DHA esterified to LPC was incorporated in the brain compared to non-esterified DHA (Thies et al., 1994). Additionally, most commercial omega-3 fatty acid supplements that are currently available, such as fish oil supplements, are in the TG

compartment (Konagai et al., 2013). Therefore, it would be interesting to see if omega-3 fatty acid supplements were given in the LPC form, would it increase the uptake of DHA into the brain more than consuming omega-3 fatty acid supplements in the TG form. Additionally, in the main study cognitive tests were done to the participants at baseline and post omega-3 fatty acid supplements for six months. With further investigation, current results can be compared with the cognitive test results. Furthermore, as I mentioned earlier, plasma samples were collected every month as the participants were taking the omega-3 fatty acid supplements for six months. These samples are stored in the -80 °C freezer. With these samples a pharmacodynamic can be done to investigate the effect of taking omega-3 fatty acid supplements over a period of six months.

For future lipidomic analysis, to quantify lipid species from various biological samples a liquid chromatography- mass spectrometry (LC-MS) can give more precise results with less errors compared to doing a TLC for lipid separation and then quantifying with gas chromatography. LC-MS analysis has many advantages such as ability to efficiently separate lipid species, identify trace amount of lipid species, separate isomers, shorter analysis time, and less solvent usage. A lot of recent and current lipidomic research are mostly using direct infusion-MS and LC-MS. Comparatively, gas chromatography and TLC are in less usage in the lipidomic field. Once lipids are extracted from the biological sample, it can be separated into different lipid compartments using LC-MS including the separation of phospholipids chromatographically (Cajka & Fiehn, 2014). In the current study, we used a second TLC for the separation of phospholipids. By this the sensitivity of our results decreased and the variation between samples were wider. Therefore, using LC-MS would improve the sensitivity and decrease variation between samples and lipid species. Ionization techniques will vary depending on which lipids are being analyzed. Positive mode electrospray ionization is more commonly used in LC-MS as it can effectively ionize an extensive variety of lipids. However, negative mode ionization is much better for phospholipids like phosphatidylinositol, phosphatidylserine and phosphatidic acid. Additionally, when comparing LC-MS to LC-MS/MS, LC-MS/MS would give results with higher resolution and more precision. The target molecule would be

filtered twice, separating the target ions from interferences (Gallien, Duriez, Demeure, & Domon, 2013). Therefore, for future lipidomic analysis usage of LC-MS/MS would be better to obtain results faster and with more sensitivity than using TLC to separate lipid compartments.

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